THE BEHAVIOUR AND ECOLOGY OF THE ZEBRAFISH, *Danio rerio*

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

Rowena Spence
Department of Biology
University of Leicester

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Hi,

I'm writing to inquire about whether anyone is aware of papers or researchers studying zebrafish ecology or evolutionary ecology. Given all the fantastic work produced by developmental and molecular geneticists on this system, it surprises me that no evolutionary ecologists seem to be working on these fish in their natural populations. It seems like the vertebrate answer to using drosophila as a study system for evol. ecology. Are you aware of any work being done or references I could go to on such a topic?

thanks
Dan Bolnick

message posted to www.bio.net
September 1999

ABSTRACT

Though the zebrafish is an important laboratory model organism, little is known about its natural ecology, and few studies have been conducted on wild populations. It is a small, shoaling cyprinid, native to the floodplains of the Indian subcontinent. A survey conducted in Bangladesh showed that it is most abundant in shallow lakes, ponds and ditches, often associated with rice cultivation. Length-frequency analysis indicates that the zebrafish is an annual species, recruitment being linked to the monsoon season. Its diet, based on gut content analysis, consists primarily of zooplankton and insects.

Zebrafish are group spawners and egg scatterers, though females are choosy with respect to sites for oviposition and males defend territories around such sites. The reproductive success of both females and males is affected by density; females produce smaller clutches at higher densities; at low densities territorial males achieve greater reproductive success than non-territorial males, but at higher densities they are no more successful than non-territorials. Female mating preferences, when assessed in the absence of male-male competition, do not correspond to male dominance. The opportunity for selection appears to be weak in zebrafish.

Zebrafish show innate and learned preferences for visual cues, both in social and foraging contexts. They shoal preferentially with their own colour pattern, but when individuals are cross-reared with groups of an alternative colour pattern, they prefer to shoal with fish of the appearance with which they were reared; there appears to be a strong learned component to species recognition. In a foraging context, zebrafish display both innate and learned colour preferences. When fish were reared on diets consisting of different colours, each group respond most strongly to red, although there was also an effect of conditioning.

This project provides a basis for further studies linking adaptive behaviour with gene expression in zebrafish.

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CHAPTER ONE: INTRODUCTION

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Aims of the project

The zebrafish, *Danio rerio* (Hamilton), is an important model organism in developmental genetics and neurophysiology (Grunwald & Eisen 2002) and potentially for behavioural, ecological and evolutionary biology. However, while there are numerous protocols for the laboratory use of zebrafish (Westerfield 2000; Detrich *et al.* 1999a; Nüsslein-Volhard & Dahm 2002), little is known about its natural ecology and behaviour and few studies have been conducted on wild populations. The aim of this project was to address this gap in our knowledge by:

- Describing their basic ecology, including habitat preferences, assemblage, diet, growth and recruitment.
- Describing their mating system, including male competition, female mating preferences, measuring the opportunity for sexual selection, and comparing the behaviour of wild-caught and laboratory zebrafish.
- Investigating innate and learned responses to visual cues in social and foraging contexts.

Structure of thesis

This thesis is presented as a set of nine papers submitted to peer-reviewed scientific journals. Paper A reviews the current state of knowledge of the ecology and behaviour of the zebrafish, including taxonomy, natural ecology, reproductive ecology, social behaviour and learning. It explains the importance of the zebrafish as a vertebrate model organism and discusses its potential as a model for studying the genetic basis of behaviour. This paper forms the introduction to the thesis and includes reference to the experimental work presented in subsequent chapters. Chapter two focuses on the natural ecology of the zebrafish. Paper B presents the results of a field survey on the distribution and habitat preferences of the zebrafish in two regions of Bangladesh, while Paper C presents data on diet, growth and mortality of wild zebrafish, based on a 12-month sampling programme. Chapter three comprises four papers that address the reproductive ecology of the zebrafish. Paper D describes the effects of density and operational sex ratio on male behaviour and female oviposition. It includes the first description of territorial mating behaviour among male zebrafish. Paper E presents the results of a microsatellite parentage analysis of a subset of the offspring from the experiment in paper D, enabling the reproductive success of territorial males to be quantified and the opportunity for sexual selection in zebrafish to be estimated. Paper F investigates female mating preferences in the absence of male-male competition, pairing females sequentially with males of different dominance ranks and using egg production as a measure of mating preference. Paper G examines how oviposition decisions are affected by spawning site quality and compares the spawning behaviour of domesticated zebrafish in the laboratory with that of wild fish in a field-based study in Bangladesh. Chapter four considers learned and innate responses to visual cues in different contexts. Paper H explores social preferences for different colour patterns, using cross-rearing to test the prediction that preferences are learned. Paper I examines the relative contributions of learning and an innate bias on colour preferences in a foraging context. Chapter five summarises the major findings of the project and discusses possible future directions for research.

Paper A: Review of the ecology and behaviour of the zebrafish

This paper has been submitted to: *Biological Reviews of the Cambridge Philosophical Society.*

Authors: Rowena Spence and Carl Smith

Abstract

The zebrafish is an important model organism in developmental genetics and neurophysiology but little is known about its natural ecology and behaviour. It is a small, shoaling cyprinid, native to the flood-plains of the Indian subcontinent, where it is found in shallow, slow-flowing waters. Zebrafish are group spawners and egg scatterers, although females are choosy with respect to sites for oviposition and males defend territories around such sites. Laboratory studies of zebrafish behaviour have encompassed shoaling preferences, foraging dynamics and learning. These studies are discussed in relation to the suitability of the zebrafish as a model for behavioural genetics.

INTRODUCTION

The zebrafish as a model organism

The zebrafish is one of the most important vertebrate model organisms in genetics, developmental biology, neurophysiology and biomedical research (Grunwald & Eisen 2002; Eisen 1996; Vascotto *et al.* 1997). It has a number of intrinsic attributes that make it particularly tractable to experimental manipulation. It is a small, robust fish, so large numbers can be kept easily and cheaply in the laboratory. It breeds all year round; females can spawn every 2-3 days and a single clutch may contain several hundred eggs. Generation time is short, typically 3-4 months, making it suitable for selection experiments. Zebrafish eggs are relatively large, (0.7 mm in diameter at fertilisation) and optically transparent, the yolk being sequestered into a separate cell. Furthermore, fertilisation is external so live embryos are accessible to manipulation and can be monitored through all developmental stages under a dissecting microscope (Kimmel *et al.* 1995). Development is rapid, with precursors to all major organs being acquired within 36 h and larvae displaying food seeking and active avoidance behaviours within five days post fertilisation (Kimmel *et al.* 1995).

A popular aquarium species, the zebrafish has been used in developmental biology for many years (e.g. Creaser 1934). Its current prominence as a model organism stems from the work of Streisinger (1981), who pioneered its use to apply molecular genetics to the study of vertebrate embryology, and Kimmel (1989, 1993; Kimmel *et al.* 1990), who published detailed descriptions of cell differentiation and nervous system organisation (for review see Grunwald & Eisen 2002). The zebrafish was the subject of the first large-scale random mutagenesis screens to be conducted in a vertebrate species (Granato & Nüsslein-Volhard 1996). These screens, conducted in 1996 in Boston (Driever *et al.* 1996) and Tübingen (Haffter *et al.* 1996a), generated over 4,000 mutations and led to the identification of over 400 genes controlling vertebrate development. Since then there have been numerous technological advances (for review see Postlethwait & Talbot 1997; Patton & Zon 2001; Udvadia & Linney 2003; Chen & Ekker 2004; Guo 2004), culminating in the zebrafish genome project, based at the Sanger Institute in Cambridge, which began in 2001 and will shortly be completed (www.sanger.ac.uk). The zebrafish is becoming increasingly important in biomedical research (Dooley & Zon 2000; Shin & Fishman 2002). Its strength as a model organism is that as a vertebrate it is more comparable to humans than invertebrate model species such as the fruitfly, *Drosophila melanogaster* (Postlethwait *et al.* 1998; Barbazuk *et al.* 2000), while being more tractable to genetic and embryological manipulation than traditional biomedical model species such as mice, *Mus musculus,* in which such procedures are both complicated and costly.

Over 400 labs worldwide now routinely use the zebrafish in fundamental and applied research (ZFIN) and there is an increasing interest in its use as a model for understanding the genetic basis of behaviour (Gerlai 2003; Guo 2004; Miklósi & Andrew 2006). Despite this interest, it has attracted little attention from the behavioural ecology community. Furthermore, little is known about its natural ecology and few studies have been conducted on wild populations. Most research lines are the product of many generations in captivity which is likely to have resulted in selection for reproductive capacity, while relaxing selection for other traits, such as predator avoidance (Robison & Rowland 2005; Wright *et al.* 2006a). Thus, it is not clear to what extent and in what respect domesticated strains may differ from wild fish, nor how much inter-population variation exists in nature. The last comprehensive review of the biology of the zebrafish was by Laale (1977), the main focus of which was on physiology. The purpose of this paper is to review the current state of knowledge of the ecology and behaviour of the zebrafish. The term behaviour is used not in the sense of simple, reflexive responses to stimuli but rather for complex behavioural syndromes such as those involved in social and reproductive behaviour.

Scope of the review

The review starts with a summary of the taxonomic status of the zebrafish, which has recently undergone revision, together with a brief description of its external appearance, and a summary of the main laboratory strains. The next section reviews what is known of its natural ecology, including distribution, habitat, natural diet, growth and mortality, assemblage, predators and parasites. Growth and mortality in both wild and domesticated zebrafish are compared although we do not attempt to review zebrafish development, this subject having been extensively dealt with elsewhere (Barinaga 1990, 1994; Detrich *et al.* 1999b; Kimmel 1989; Kimmel *et al.* 1990). The next section focuses on zebrafish reproductive ecology, including spawning behaviour, which is largely known from studies on domesticated strains although some information is available on wild fish. The majority of behavioural studies on zebrafish concern aggregation and shoaling and are reviewed in the next section. The review then turns to what is known about zebrafish cognitive behaviour, including learning and innate biases. While zebrafish genetics is also outside the scope of this review, the final section reviews the growing number of studies that have focused on zebrafish as a potential model for behavioural genetics.

TAXONOMY

Taxonomic status

The zebrafish belongs to the family of freshwater fishes Cyprinidae, the most species rich vertebrate family (Nelson 1994). There are currently approximately 44 danionin species (Fang 2001), distributed throughout South and southeast Asia, their highest species diversity in north-eastern India, Bangladesh and Burma (Barman 1991). The name *Danio* derives from the Bengali name "*dhani*", meaning "of the rice field" (Talwar & Jhingran 1991). Danios are included in the subfamily Rasborinae (Howes 1991). They are characterised by small size

(<120 mm total length), the presence of a 'danionin notch', in the ventromedial margin of the dentary, and a distinctive colour pattern based on alternating dark and light horizontal stripes, which may be broken up into blotches or bars.

D. rerio was first described by Francis Hamilton, a surgeon with the British East India company, stationed principally in West Bengal at the beginning of the 19th Century. He published *An Account of the Fishes Found in the River Ganges and its Branches* in 1822 which included ten *Danio* species. *D. rerio* was later assigned to the subgenus *Brachydanio,* together with the other small *Danio* species with short dorsal fins and a reduced lateral line, *Danio* being reserved for the larger species of the group (Weber & de Beaufort 1916). *Danio* and *Brachydanio* were synonomised by Barman (1991) as there were no diagnostic characters that reliably separated the two groups. The first molecular phylogeny of the group was produced by Meyer *et al.* (1993; 1995) based on 16S and 12S mitochondrial DNA for nine species. This analysis showed *Danio* was monophyletic with two subclades that were either deep-bodied or slender-bodied. Subsequent molecular studies (McClure 1999; Parichy & Johnson 2001) supported this distinction, as did a combined molecular and morphological study by Sanger & McCune (2002). Moreover, Parichy & Johnson (2001) showed that hybrid viability and fertility among *Danio* species largely corresponded to the relationships inferred from molecular data.

However, a more complete phylogeny based on morphological analysis proposed that *Danio* was paraphyletic, the slender-bodied clade being more closely related to *Esomus* than to the deep-bodied clade (Fang 2003). This study included 13 *Danio* species together with an additional eight closely related genera, and was based on 38 morphological characters. The deep-bodied clade has now been assigned the distinct generic name of *Devario*, and includes most of the striped and barred danios (currently 35 valid species), with *Danio sensu stricto,* (including *D. rerio*) restricted to nine species (Fang 2003). The two genera (*Devario* and

Danio) cannot be reliably distinguished on the basis of proportional measurements alone, as there is considerable intraspecies variation, mature females typically being deeper bodied than males or juveniles. Although *Devario* tend to be larger, one of the large species, *Danio dangila,* is included in *Danio* (Fang 2001; Parichy & Johnson 2001). However, the two genera are ecologically quite distinct, *Devario* spp. occurring in hill streams with clear running water, while the true *Danio* spp. are confined to lowland areas, typically inhabiting turbid streams and pools (Fang 2001).

Appearance and morphology

D. rerio rarely exceeds 40 mm Standard Length (from the tip of the snout to the origin of the caudal fin (SL)). Its body shape is fusiform and laterally compressed, with a terminal oblique mouth directed upwards. The lower jaw protrudes further than the upper and the eyes are central and not visible from above. The diagnostic features for the species are an incomplete lateral line extending to the pelvic fin base, two pairs of barbels and five to seven dark blue longitudinal stripes extending from behind the operculum into the caudal fin (Barman 1991). The anal fin is similarly striped, while the dorsal fin has dark blue upper edge, bordered with white. The colour pattern comprises three types of pigment cell, dark blue melanophores, gold xanthophores and iridescent iridophores. Developmentally, two stripes first form centrally with subsequent stripes being added sequentially above and below (McClure 1999). As with many teleosts, the melanophores can be concentrated or dispersed in response to stimuli, which functions both in camouflage, melanophores aggregating and dispersing in response to light intensity (Guo 2004) and signalling, fish typically darkening during aggressive display (Gerlai 2003; Larson *et al.* 2006). Males and females are of similar colouration, although males tend to have larger anal fins with more yellow colouration (Laale 1977; Schilling 2002). The sex of juveniles cannot be reliably distinguished without dissection and while gravid females have a more rounded body shape, the most reliable diagnostic feature is the presence of a small genital papilla in front of the anal fin origin.

Laboratory strains

Zebrafish used for mutagenesis and screening are from lines bred in laboratories for many generations in order to maintain a stable genetic background. They are also "cleaned up"; i.e. bred selectively to remove embryonic lethal mutations. The main currently recognised wild-type lines from the Zebrafish International Resource Center are shown in Table I. For details of mutant lines see http://zfin.org.

The "leopard" danio, which displays a spotted colour pattern instead of stripes, was originally thought to be a separate species, described as *Brachydanio frankei* (Meinken 1963). However, neither molecular nor morphological analyses have differentiated between the two (Fang 1998; Meyer *et al.*1993) while hybrids were shown to produce fertile progeny (Petrovicky 1966; Parichy & Johnson 2001). The leopard danio is now known to be a spontaneous mutation of the wild-type *D. rerio* colour pattern (Haffter *et al.*1996b), with homozygotes displaying a spotted pattern, while heterozygotes have a disrupted stripe pattern. Leopard danio mutants are primarily bred for the aquarium trade but also occur in nature (R. Spence, pers. obs.). Another aquarium variant is the "longfin" *D. rerio,* which is another dominant homozygous mutation resulting in elongated fins (Plaut 2000).

Pigment patterns in *Danio* spp.

Comparison of pigment patterns among *Danio* species has provided insights into their evolutionary relationships. Larval *Danios* of different species form an identical pattern, which only differentiates into the adult pattern in about the third week of development (McClure 1999). Interestingly, several *D. rerio* pigment pattern mutations resemble other *Danio* species (Parichy & Johnson 2001). This remarkable concurrence in appearance raises the possibility that the alleles expressed by zebrafish colour mutants are the same as those expressed in

Table I. Wild-type zebrafish lines listed by the Zebrafish International Resource Center

Name	Description
AB	Derived from two lines purchased by George Streisinger from pet shops in late 1970s. The currently used line *AB was derived from the original AB line in 1991-1992 by parthenogenesis.
AB/Tübingen	Hybrid of AB and Tübingen wild-type lines.
Darjeeling	Collected in Darjeeling in 1987 and sent to Monte Westerfield at Oregon. A much faster swimmer than other wild-type strains. Used extensively for mapping as it contains many polymorphic markers.
Ekkwill (EKW)	From Ekkwill breeders in Florida and maintained in the Grunwald lab, University of Utah.
Hong Kong	Stock obtained from a Hong Kong fish dealer.
HK/AB	Hybrid of Hong Kong and AB wild-type lines.
HK/Sing	Hybrid of Hong Kong and Singapore wild-type lines.
India	Stock obtained from expedition to Darjeeling (date uncertain).
Indonesia	Stock obtained from Indonesian fish dealer.
Nadia	Wild caught about 40 miles east of Calcutta. The fish were collected from stagnant ponds and flood plains. Imported in 1999 by a wholesaler in Oregon. Established in the Oregon laboratory from an initial group of ten individuals.
Singapore	Stock obtained from a Singapore fish dealer.
Tübingen	Wild-type short fins. Strain used by the Sanger Institute for the zebrafish genome sequencing project.
WIK	Derived from wild catch in India (date and location uncertain) and used for genome mapping.

related *Danio* species. Consequently, these alleles may have played a role in colour pattern diversification among species (Parichy & Johnson 2001).

A spectacular array of adult pigment pattern mutants have been identified for zebrafish (Haffter *et al.* 1996b; Kelsh *et al.* 1996). Many mutant colour patterns can be attributed to a single locus, and several pigment genes have been identified at the molecular level (Lister *et al.* 1999; Kawakami *et al.* 2000). In a study of the mechanisms of colour pattern inheritance, Parichy & Johnson (2001) showed that hybrids between zebrafish and four closely related *Danio* species all expressed pigment patterns resembling that of wild-type zebrafish. These findings imply that stripes may be ancestral in *Danio* spp. Thus the zebrafish may serve as a useful model for studying the genetic and developmental basis of colour pattern evolution as a mechanism for speciation.

ECOLOGY

Distribution and habitat

The natural range of the zebrafish is centred around the Ganges and Brahmaputra river basins in north-eastern India, Bangladesh and Nepal although it also occurs in the Indus, Cauvery, Pennar, Godavari and Mahanadi river basins. In addition, it has been reported as occurring in the Krishna river basin (Talwar & Jhingran 1991) and in the states of Rajasthan, Gujarat and Andra Pradesh (river basins draining into the Arabian Sea) as well as northern Myanmar and Sri Lanka, although no location details are given (Barman 1991). The reliability of some of the records is questionable; either no specimens appear to have been collected (as in the case of records for Sri Lanka), or the specimen has been reclassified (as in the case of at least one species from Myanmar, now designated *Danio kyathit*, Fang 1998). Database records for this species should not be considered as complete (Table II, Fig. 1). However, on the basis of confirmed occurrences, the zebrafish may be widely distributed over the Indian subcontinent;

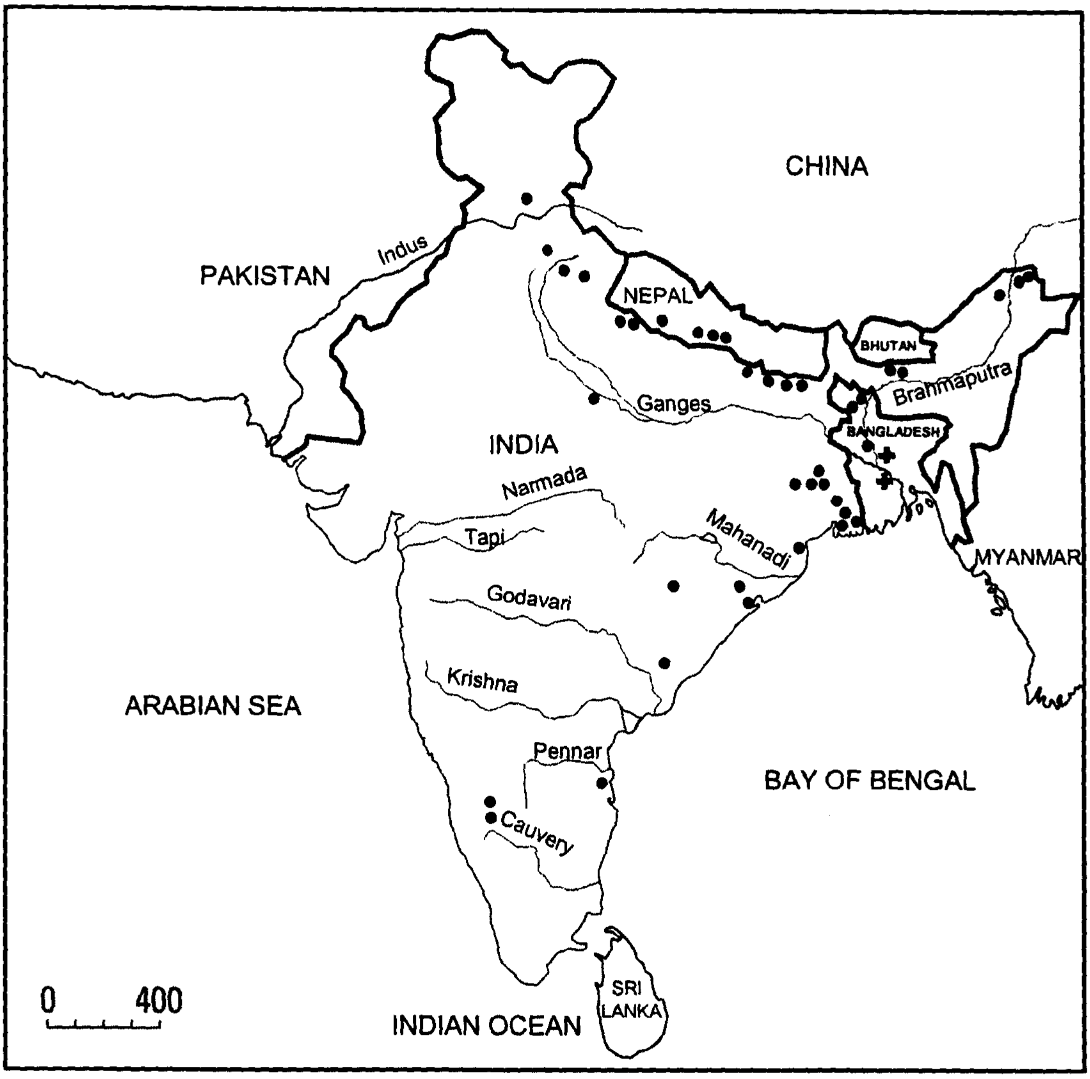

Figure 1. Natural distribution of the zebrafish. Major river systems indicated. Black dots indicate recorded occurrences. Black crosses indicate field sites sampled in paper B.

it may be overlooked in surveys on account of its small size and the fact that it has no value as a food fish, even to subsistence fishermen.

The Indian subcontinent has a monsoon climate with wide seasonal variation in the extent of freshwater habitats. Some of the major river systems, such as the Ganges, run through low-lying areas which flood extensively during the monsoon months. The floodplains are characterised by oxbow lakes and blind channels, which may have seasonal connections to the main river. In addition, these regions contain extensive areas of man-made lakes, ponds and

Table II. Reported occurrences of *Danio rerio*

Country	Year	Latitude/ longitude		Drainage	Source	Site description
Bangladesh	2005	22	90	Ganges	Spence et al. (2006a)	Ditch on campus of Khulna University, 3 m wide, <1 m deep, Secchi depth 50 cm, no vegetation. Grassy bank. Some shade.
Bangladesh	2005	22	90	Ganges	Spence et al. (2006a)	Isolated channel of R. Golamari, near Khulna. Approx. area 200 x 1500 m, 50 cm deep, Secchi depth 19 cm, vegetation at margins, silt substrate. Grassy bank. No shade.
India	1998	22	88	Ganges	Fang & Roos, Swedish Natural History Museum	About 65 km NNE of Calcutta, R. Tumapao close to Duma village, shore. Stream more than 100 m wide and > 0.7 m deep with slow to moderately flowing, moderately turbid, brownish water. Adjacent to rice-field, no vegetation on bank, vegetation in water. Silt substrate.
India	1998	24	87	Ganges	Fang & Roos, Swedish Natural History Museum	Stream on Dumka-Rampurhat road, about 7 m wide and about 1 m deep, with slow-flowing, clear, greenish water. About 5% shade. Hilly area with grasses on land and on bank, some plants in water. Silt substrate.
India	1998	24	87	Ganges	Fang & Roos, Swedish Natural History Museum	Roadside stream about 62 km from Bhagalpur on Deughar-Bhagalpur road. Small stream 3 m wide and 0.5 m deep with fast running, clear, brownish water. No shade. Hilly area without vegetation on land, bank or in water. Sand substrate.
India	1998	24	86	Ganges	Fang & Roos, Swedish Natural History Museum	Stream on Jamtara –Deughar road, about 5-8 m wide and 0.4 m deep with moderate current and clear, uncoloured water. No shade. Hilly area with grass on land, no vegetation on bank or in water. Sandy substrate
Nepal	1996	27	83	Ganges	Edds, Kansas University Ichthyology Collection	Tribeni.
Nepal	1996			Ganges	Pritchard (2001)	Shallow ditches & pond on Ranpur campus of Royal Nepal Agricultural College, Chitwan. Clear, slow or still water, silt substrate, some vegetation.
Nepal	1996	28	80	Ganges	Edds, Kansas University Ichthyology Collection	3km W of Pipariya, Shuklaa Phataa Wildlife Reserve.
Nepal	1996	28	80	Ganges	Edds, Kansas University Ichthyology Collection	Confluence of 3 rivers (Chaudhar, Bahuni, Gobraiya) at Royal Shuklaa Phantaa Wildlife Reserve.
Nepal	1996	28	80	Ganges	Edds, Kansas University Ichthyology Collection	Raj-Marg highway, 9 km E of Mahendranagar.

Table II continued

Country	Year	Latitude/ longitude		Drainage	Source	Site description
Nepal	1996	28	80	Ganges	Edds, Kansas University Ichthyology Collection	Waters of Kailali district along Raj-Marg highway.
Nepal	1996	27	84	Ganges	Edds, Kansas University Ichthyology Collection	Narayangarh.
Nepal	1996	26	86	Ganges	Edds, Kansas University Ichthyology Collection	Just downstream from irrigation dam at Phattepur.
Nepal	1996	26	86	Ganges	Edds, Kansas University Ichthyology Collection	Just upstream from R. Koshi barrage.
Nepal	1996	26	88	Ganges	Edds, Kansas University Ichthyology Collection	Bhadrapur.
Nepal	1996	26	87	Ganges	Edds, Kansas University Ichthyology Collection	Belbari.
India	1995			Ganges	McClure et al. (2006)	Tributary of R. Song, Lachiwala, Dehra Dun, UP. 1-12 m wide, 16-57 cm deep, Secchi depth > 35 cm. Substrate clay, silt, cobble, boulders. Shade 0-50%.
India	1995			Ganges	McClure et al. (2006)	Side channel of R. Pasuni, Janakikund, Banda, UP. Site characteristics as above.
India	1995			Ganges	McClure et al. (2006)	Rice paddy connected to R. Bhairab, near Bak Bungalow, Parganas, W. Bengal. Site characteristics as above.
Nepal	1975			Ganges	Roberts, California Academy of Science	Chitawan Valley, 10 miles W of Narangar.
Nepal	1975			Ganges	Roberts, California Academy of Science	Chitawan Valley, at Kasa Darbar or Dabar.
Nepal	1975			Ganges	Roberts, California Academy of Science	Chitawan Valley, including Khagari Khola, 45 miles E and slightly N of Hetaura (Hitaura) & 11 miles SSE of Narangar.
Nepal	1975			Ganges	Roberts, California Academy of Science	Chitawan Valley, low-lying mountain stream 1-2 miles S of Khoria Mohan in Someswar Hills (Hathimara Khola).

Table II continued

Country	Year	Latitude/ longitude		Drainage	Source	Site description
Nepal	1975			Ganges	Roberts, California Academy of Science	Chitawan Valley, R. Reu near confluence with R. Rapti.
Nepal	1975			Ganges	Roberts, California Academy of Science	Farm pond 1-2 km east of Kalaiya or Khailaya.
India	1949	24	85	Ganges	Choata - Nagpur Survey, University of British Columbia	R. Barakar near Tillya dam.
India	1937	22	88	Ganges	Herre, California Academy of Science	R. Ganges delta at Pulta.
India	1932	31	75	Ganges	Khan, British Museum of Natural History	Phillaur (R. Sutley), Punjab.
India	1889	23	87	Ganges	Day, British Museum of Natural History	Bengal.
Bangladesh	2005	24	90	Brahmaputra	Spence et al. (2006a)	Isolated pond, Sutiakali, near Mymensingh. Approx. area 30 x 50 m, 15 cm deep, silt substrate, vegetated. Grassy bank. No shade.
Bangladesh	2005	24	90	Brahmaputra	Spence et al. (2006a)	Isolated pond, Sutiakali, near Mymensingh. Approx. area 10 x 12 m, 40 cm deep, Secchi depth 15 cm, silt substrate, no vegetation. Grassy bank. Some shade.
Bangladesh	2005	24	90	Brahmaputra	Spence et al. (2006a)	Isolated pond, Sutiakali, near Mymensingh. Approx. area 10 x 10 m 1m deep, Secchi depth 30 cm, silt substrate, no vegetation. Grassy bank. Some shade.
Bangladesh	2005	24	90	Brahmaputra	Spence et al. (2006a)	Large semi-natural pond at Bangladesh Agricultural University field station, Mymensingh. Approx. area 105 m^2, 1m deep, Secchi depth 30 cm, silt substrate, vegetation at margins. Vegetation on bank. No shade.
Bangladesh	2005	24	90	Brahmaputra	Spence et al. (2006a)	Ditch connecting to paddy fields, Bangladesh Agricultural University field station, Mymensingh. 10 m wide, 50 cm deep, vegetation. Grassy bank. No shade.
Bangladesh	2005	24	90	Brahmaputra	Spence et al. (2006a)	Small semi-natural pond at Bangladesh Agricultural University field station, Mymensingh. Approx. area 8 x 15 m, 65 cm deep, Secchi depth 15 cm, silt substrate, vegetation. Vegetation on bank. No shade.

Table II continued

Country	Year	Latitude/ longitude		Drainage	Source	Site description
Bangladesh	2005	24	90	Brahmaputra	Spence et al. (2006a)	Channel adjacent to campus of Bangladesh Agricultural University, feeding into field station. 8 m wide, 75 cm deep, Secchi depth 15 cm, silt substrate, vegetation. Grassy bank. No shade.
India	1998	27	94	Brahmaputra	Fang & Roos, Swedish Natural History Museum	Roadside ditch by the Sessa Tinali (Sessa crossing) on Dibrugarh – Jorhat road. About 15 m wide and 0.1-0.4 m deep with stagnant, brownish water. No shade. Plain with grass on land, no vegetation on bank, vegetation in water. Silt substrate.
India	1998	27	95	Brahmaputra	Fang & Roos, Swedish Natural History Museum	About 100km SSE of Dibrugarh, small stream near R. Dilli. Stream about 2 m wide and 0.8 m deep with moderate current and yellow/brownish water. No shade. Plain with grass on land and bank, green algae in water. Silt substrate.
Bangladesh	1997			Brahmaputra	Pritchard (2001)	Small shallow pools in a dry river bed and an adjacent spring-fed pond in a village of the Santal tribal group near to the India-Bangladesh border. Some vegetation.
Bangladesh	1997			Brahmaputra	Pritchard (2001)	Artificial concrete channel at Northwest Fisheries, Saidpur, Bangladesh. Still, extremely turbid water. No vegetation.
Nepal	1997			Brahmaputra	Pritchard (2001)	Pond 10 km N of Tangail, next to Tangail-Madhupur highway. Clear water, silt substrate and vegetation. 1.5m deep.
India	1957			Brahmaputra	v. Maydell, Zoological Museum of Hamburg	Raimona, R. Janali.
India	1957	26	93	Brahmaputra	v. Maydell, Zoological Museum of Hamburg	Kaziranga, Mikir-Hills.
India	1956	30	77	Brahmaputra	v. Maydell, Zoological Museum of Hamburg	Dharmawalla (Siwalik), R. Asan.
India	1956	28	81	Brahmaputra	v. Maydell, Zoological Museum of Hamburg	Nishangara, Varei-Bach.
India	1956	25	91	Brahmaputra	v. Maydell, Zoological Museum of Hamburg	Umsa, W. Assam, Khasi Hills.
India	1956	25	91	Brahmaputra	v. Maydell, Zoological Museum of Hamburg	Garampani, Assam, R. Kopili.

Table II continued

Country	Year	Latitude/ longitude		Drainage	Source	Site description
India	1938	27	88	Brahmaputra	Hora, California Academy of Science	Kalimpong Duars & Siliguri Terai.
India	1993			Indus	Dutta (1993)	Gadigarh stream, Jammu.
India	1987			Cauvery	Roberts, California Academy of Science	NW/WNW of Mysore.
India	1955			Cauvery	v. Maydell, Zoological Museum of Hamburg	Jog-Falls, Sharavati-R.
India	1934			Cauvery	Sundberg, Swedish Natural History Museum	Mysore.
India	1868	13	80	Pennar	Day, British Museum of Natural History	Madras.
India	1961	19	81	Godavari	Ross & Cavagnaro, California Academy of Science	9 miles north of Pharasgro, in pond among water plants.
India	1983	21	86	Mahanadi	Parshall, British Museum of Natural History	R. Salane.
India	1940	21	81	Mahanadi	Herre, California Academy of Science	Bisrampur.
India	1889	20	84	Mahanadi	Day, British Museum of Natural History	Orissa.

irrigation channels, constructed for fish and rice cultivation. There is a wide range of temperatures within the natural range of zebrafish, from 5 °C in winter to 34 °C in summer.

Zebrafish have typically been described as inhabiting slow-moving or standing water bodies, the edges of streams and ditches, particularly adjacent to rice-fields (Sterba 1962, Talwar & Jhingran 1991; Jayaram 1999). However, they are also reported as inhabiting rivers and hill streams (Daniels 2002). This inconsistency in habitat preference probably results from the taxonomic confusion between *Danio* and *Devario* (Fang 2001). Two surveys have described their habitat systematically; McClure *et al.* (2006) captured zebrafish in three sites in the Ganges drainage in India, while Spence *et al.* (2006a, paper B) captured them in nine sites in the Ganges and Brahmaputra drainages in Bangladesh. In both studies, zebrafish were found to occur in shallow waterbodies with a Secchi depth of ~30 cm, frequently in locations with little canopy, aquatic vegetation and with a silty or gravel substrate. Zebrafish appear to be a floodplain rather than a true riverine species. They are most commonly encountered in shallow ponds and standing water bodies, often connected to rice cultivation. This association with rice cultivation may relate to the use of fertilisers which may promote the growth of zooplankton, a major component of the zebrafish diet (Spence *et al.* in press, paper C). Spence *et al.* (2006a, paper B) found no zebrafish either in rivers or temporary creeks that opened during the monsoon season. Where zebrafish are found in streams and rivers, these typically have a low flow regime and zebrafish tend to be found at the margins (McClure *et al.* 2006). Observations of their vertical distribution indicated that they occupy the whole of the water column and occur as frequently in open water as amongst aquatic vegetation (Spence *et al.* 2006a, paper B).

Diet

The zebrafish is omnivorous, its natural diet consists primarily of zooplankton and insects, although phytoplankton, filamentous algae and vascular plant material, spores and invertebrate eggs, fish scales, arachnids, detritus, sand and mud have also been reported from gut content analysis (Dutta 1993; McClure *et al.* 2006; Spence et al, in press, paper C). The majority of insects identified in these studies were aquatic species, or aquatic larval forms of terrestrial species, particularly dipterans, and it has been suggested that the zebrafish may have some value in mosquito control (Dutta 1993). The high proportion of planktonic items in their diet indicates that zebrafish feed chiefly in the water column. However, terrestrial insects and arachnids are also consumed, suggesting surface feeding, while the presence of inorganic elements and detritus suggest they also feed from the substrate. In a study based on sampling over 12 months, dietary composition appeared to differ significantly among months although no clear seasonal pattern was apparent (Spence *et al.* in press, paper C). Additional data are required to determine the extent to which food items in the gut of zebrafish reflect selectivity on the part of the fish as opposed to seasonal availability of different prey.

Growth and mortality

Zebrafish growth rate is rapid during the first three months, after which it decreases, approaching zero by about 18 months (Spence *et al.* in press, paper C). Growth rates are considerably higher in domesticated strains than in nature (Robison & Rowland 2005), possibly due to inadvertent selection for rapid growth or as a consequence of a better diet in captivity. Eaton and Farley (1974a) reported an annual growth rate of 183 mm y^{-1} during the first 45 days of development, compared to 72 mm y^{-1} during the first two months in nature (Spence *et al.* in press, paper C). Domesticated strains also appear to achieve a larger body size than wild fish (Wright *et al.* 2006a). A length-frequency analysis based on sampling over 12 months from a lake population in Bangladesh showed that the majority of fish measured 25 mm after one year. The maximum SL observed was 35 mm (Spence *et al.* in press, paper C), which is comparable to the typical range observed in domesticated populations. The size difference may be partly due to genetic factors (Wright *et al.* 2006a, 2006b), with selection for fast growth and high fecundity among laboratory fish, but it also reflects rearing conditions; in the laboratory, F_1 fish from natural populations often grow to 35 mm SL (R. Spence, pers. obs.). Females tend to be larger than males both in domesticated and wild populations (Eaton & Farley 1974a, 1974b; Spence *et al.* in press, paper C). The extent of variation in growth rates and body size among wild populations is unknown.

The zebrafish appears to be primarily an annual species in nature, the spawning season commencing just before the onset of the monsoon (Spence *et al.* in press, paper C). Length-frequency analysis showed two distinct age classes during the summer months, representing reproductively mature 1+ year fish and a cohort of 0+ year fish. Thus, the main period of rapid growth takes place during the monsoon months (June-September), a period of high temperatures (up to 34 °C) and food availability (Talling & Lemoalle 1998).

Gerhard *et al.* (2002) reported a mean life span of domesticated zebrafish of 42 months, with the oldest individual surviving for 66 months. However, instances of spinal curvature, a phenotype commonly associated with senescence (Gerhard *et al.* 2002), become apparent in domesticated and wild zebrafish after their second year in captivity (R. Spence, pers. obs.). Spinal curvature was not observed in a wild population (Spence *et al.* in press, paper C) and it is likely that fish die in natural populations before this condition develops.

Assemblage

Where zebrafish are found, they tend to be among the most abundant species (McClure *et al.* 2006; Spence *et al.* 2006a, paper B). Spence *et al.* (2006a, paper B) captured a total of 25 species from nine families that co-occurred with zebrafish over their range in Bangladesh. These were primarily small (< 25 cm total length) indigenous species. Such species represent potential competitors of zebrafish. Zebrafish were often observed shoaling together with the flying barb *Esomus danricus*, another abundant cyprinid of similar size and appearance which belongs to a sister group of *Danio* (Fang, 2003).

McClure *et al.* (2006) reported significant differences in the characteristic temperature, pH and current speed of the habitats in which different danionin species occurred. The other danionin species found with zebrafish were *Devario devario* and *D. aequipinnatus*, although the *Devario* species typically inhabited faster flowing water whereas zebrafish were captured in the margins of streams and rivers. This corresponds with Fang's (2001) finding that the two genera occupy different microhabitats.

Predators

The most common predatory taxa captured by Spence *et al.* (2006a, paper B) were snakeheads, *Channa* spp., and the freshwater garfish, *Xenentodon cancila* (Hamilton 1822), although their sampling protocol may have failed to capture other potential predators such as nocturnal catfishes. Avian predators such as the Indian pond heron, *Ardeola grayii* and the common kingfisher, *Alcedo atthis,* are also ubiquitous in the floodplains of the Indian subcontinent and may feed on *D. rerio.*

Laboratory studies have shown that zebrafish display fright reactions in response to both visual and olfactory cues associated with predators. Dill (1974a, 1974b) used both living (largemouth bass, *Micropterus salmoides*) and model predators to investigate zebrafish escape responses. The distance at which the response was elicited depended on the predator's size and its approach velocity. Reactive distance did not differ significantly between living and model predators, although escape velocity was higher with living predators. Over repeated trials on successive days, zebrafish responded earlier and flight distance increased. No decline in response was detected when zebrafish were retested after a 10-day break. This effect may be an example of secondary reinforcement; as the predator's approach was associated with a negative experience, the fish began to respond before the initial threshold was reached.

In common with other ostariophysian fishes, zebrafish show alarm behaviours in response to a pheromone that is released as a result of injury to the epidermal cells (Wisenden *et al.*

2004). Alarm behaviours include an increase in shoal cohesion and either agitated swimming or freezing on the substrate, a decrease in feeding rate and increase in aggression. These behaviours have been interpreted as having an anti-predator function. Rehnberg & Smith (1988) demonstrated that isolated fish showed an alarm response to water containing alarm substance so the response is independent of the presence of conspecifics.

Parasites

Little is known about the natural parasite fauna of zebrafish, or the role parasites play in their behaviour and ecology. In laboratory stocks, infection by the microsporidian *Pseudoloma neurophila* is common (Spitzbergen & Kent 2003). It infects the central nervous system, cranial and spinal nerves, and skeletal muscle of zebrafish, causing emaciation, ataxia and spinal malformations. It is not clear whether vertical transmission of this parasite can occur in zebrafish. Captive zebrafish have also been subject to infection by the nematode *Pseudocapillaria tomentosa*, which infects the gut; symptoms include inflammation, emaciation and intestinal carcinomas (Kent *et al.* 2002). *P. tomentosa* can be transmitted directly and infects entire laboratory colonies.

REPRODUCTIVE BEHAVIOUR

Spawning cycle

Much of the scientific literature on zebrafish reproduction has been concerned with how best to maximise the supply of eggs (reviewed by Laale 1977) and, until recently, almost nothing was known about the reproductive ecology of wild zebrafish. In zebrafish, all gonads initially develop as ovaries, which in males start to differentiate at approximately 5-7 weeks of development (10-15 mm TL) through an intersexual stage, finally developing into normal testes by approximately the third month of development (12-17 mm TL), depending on strain and rearing conditions (Devlin & Nagahama 2002; Maak & Segner 2003). Based on samples collected over 12 months from a population in Bangladesh, sex ratios in nature appear to be 1:1 (Spence *et al.* in press, paper C).

In the laboratory, domesticated zebrafish strains breed all year round whereas in nature spawning is more seasonal. However, larger females collected in January (outside the main spawning season) have been found to contain mature ova, indicating that reproduction may not be cued by season, but may instead be dependent on food availability (Spence *et al.* 2006a, paper B). Further, reproductive maturity appears to be related to size rather than age; wild and domesticated zebrafish appear to reach reproductive maturity at similar sizes, despite their difference in growth rates. Eaton and Farley (1974a) showed that domesticated zebrafish reared at 25.5 °C reached maturity after 75 days, when females were 24.9 mm SL and males 23.1 mm. In laboratory conditions, F_1 wild zebrafish also reached reproductive maturity at approximately 23 mm SL (R. Spence, pers. obs.).

Pairs of zebrafish left together continuously spawn at frequent but irregular intervals (Eaton & Farley 1974), and a single female may produce clutches of several hundred eggs in a single spawning. While some earlier studies (eg. Hisaoka & Firlitt 1962; van den Hurk *et al.* 1987) have reported that the female reproductive cycle is 4-5 days, there is ample evidence to the contrary. In a study by Spence & Smith (2006, paper F) inter-spawning intervals ranged from 1-6 days, with a mean of 1.5 days, producing clutches ranging from 1 to over 700 eggs, with a mean of 185 (± SD 149). Clutch size correlated positively with both female body size and inter-spawning interval. Eaton & Farley (1974) reported that inter-spawning interval increased with age, from a mean of 1.9 days in 12-month old fish to 2.7 days three months later. Clutch size also increased over this period from a mean of 158 to 195. No equivalent data are available for wild zebrafish, but inter-spawning intervals tend to be greater and clutch sizes smaller than domesticated strains, which is consistent with their slower growth and smaller size (R. Spence, pers. obs.).

Histological studies have shown that ovulation and oviposition in zebrafish take place only when they mate (Hisaoka & Firlitt 1962). Free oocytes were not present in the central lumen of the ovaries or the oviducts of specimens fixed either before or immediately after spawning, even when females were kept together with males. In specimens fixed during oviposition, oocytes were present in the oviduct but not in the lumen, all mature ova are thus released in a single spawning bout. Ovulation is dependent on female exposure to male gonadal pheromones; male holding water, testis homogenates and testis fractions containing steroid glucuronides, will induce ovulation but fail to do so in females rendered anosmic by cauterising the nasal epithelium (van den Hurk & Lambert 1983; van den Hurk *et al.* 1987). Eaton & Farley (1974) showed that exposure to a male for 7 h in the afternoon was sufficient to enable eggs to be stripped from females the following morning. However, eggs were never obtained from isolated females more than once in any 5-day period after exposure to a male. Interestingly, despite the fact that egg production is non-continuous, females exposed to male pheromones for several days prior to spawning produce more eggs, of higher quality, than females isolated for several days (Gerlach 2006). This effect could be a consequence of the concentration of pheromones to which they are exposed. Bloom & Perlmutter (1977) showed that both sexes produce pheromones that function as inter- and intra-sexual attractants, and have different effects at different concentrations. For both sexes, the intra-sexual response is elicited at a lower concentration than the inter-sexual response. However, females were repelled by female holding water with a higher concentration of pheromone.

Eggs are non-adhesive and demersal, with a diameter of approximately 0.7 mm. They are released directly onto the substrate with no prior preparation and there is no parental care. Eggs become activated on contact with water and even in the absence of sperm, undergo a series of programmed developmental steps. Unfertilised eggs develop a perivitilline space but fail to develop beyond the first few cleavages (Lee *et al.* 1999). Hatching takes place between

48-72 h at 28.5 °C, depending on the thickness of the chorion and the muscular activity of the embryo inside, both of which can vary within any group of embryos (Kimmel *et al.* 1995). Immediately after hatching the larvae (measuring ~3mm) attach to hard surfaces by means of small secretory cells in the epidermis of the head (Laale 1977). Attachment at progressively higher levels enables them to reach the surface to which they need to gain access in order to inflate their swim bladders (Goolish 1999). This process occurs after about 72 h post fertilisation, whereupon swimming, feeding and active avoidance behaviours commence (Kimmel *et al.* 1995).

Mating behaviour

It is well known that spawning in domesticated zebrafish is influenced by photoperiod (Breder & Rosen 1966). Zebrafish show a distinct diurnal activity pattern, synchronised with the light/dark and feeding cycles. The first activity peak occurs immediately after illumination with two further peaks in the early afternoon and the last hour of light (Baganz *et al.* 2005; Plaut 2000). Spawning activity coincides with the first activity peak and usually commences within the first minute of exposure to light following darkness, continuing for about an hour (Darrow & Harris 2004). Recent observations have shown that spawning in zebrafish under natural conditions also commences at dawn (Spence *et al.*, paper G). Extended day length may be a contributory factor in the seasonal onset of spawning in nature. However, although spawning is largely limited to a short period at dawn, fish also spawn at other times of the day. It was noted by Breder & Rosen (1966) that adding a dash of cold water to aquaria can encourage spawning in zebrafish. Thus, it may be that a drop in water temperature or an increase in water level may be additional cues used by zebrafish. Wild zebrafish spawn during periods of heavy rain (R. Spence, pers. obs.). Notably, wild-caught zebrafish held in captivity are more likely than domesticated strains to spawn at times other than first light (R. Spence, pers. obs.).

Courtship behaviour in zebrafish consists of a male chasing the female rapidly around aquaria, often nudging her flanks with his snout and attempting to lead her to a spawning site (see below), swimming around or in front of her in a tight circle, or figure of eight, with his fins raised. If she does not follow, he may alternate between circling the female and swimming back and forth between the female and the spawning site. Once over a spawning site he swims closely alongside the female, spreading his dorsal and caudal fins around her so that their genital pores are aligned, and may oscillate his body at high frequency and low amplitude. This behaviour triggers oviposition in the female and sperm is released simultaneously. This sequence of behaviours is repeated throughout the spawning period, females releasing between 5-20 eggs at a time. Male courtship behaviour peaks in the first 30 minutes and although it continues for about an hour, few females extrude eggs after the first 30 minutes (Darrow & Harris 2004). Wild zebrafish display the same courtship and territorial behaviours during spawning as have been described in the laboratory (Spence *et al.*, paper G). Courtship involves males actively pursuing females, who utilize the whole of the water column, alternately swimming towards the surface and then diving steeply down to the substrate. Small groups of 3-7 fish usually take part in these chases.

Courtship behaviour in the male is triggered by female pheromones. In a study by van den Hurk & Lambert (1983) males, but not females, were attracted to ovarian extracts injected into the aquarium. Anosmic males failed to court females while control males only courted females that had ovulated. Further, anosmic males were extremely aggressive, suggesting that ovarian pheromones also inhibit aggression which is common in both sexes during foraging.

Zebrafish typify a basic mating pattern common to many cyprinid fishes; they are group spawners and egg scatterers (Breder & Rosen 1966). Females will spawn directly onto a bare substrate, but when provided with an artificial spawning site, such as a plastic box filled with marbles, will preferentially use it for oviposition (Spence & Smith 2005, paper D). Some male zebrafish are territorial during mating (Spence & Smith 2005, paper D). Both territorial and non-territorial males show the same courtship behaviour but whereas non-territorial males pursue females, territorial males confine their activities to within a few body lengths of a spawning site and chase other males away when they try to approach. A study by Spence & Smith (2005, paper D) examined the effects of manipulating density and sex ratio on the behaviour of these territorial males. Aggression rates increased as a function of density. However, while courtship behaviour increased with density under a female-biased sex ratio, when the sex ratio was male biased courtship rate decreased relative to that observed at low densities. A subsequent microsatellite parentage analysis showed that the reproductive success of territorial males was also density dependent (Spence *et al.* 2006b, paper E). At low densities territorial males sired significantly more offspring than non-territorial males. However, at high densities territorial males were no more successful than non-territorials. Thus male zebrafish display two distinct mating tactics, territorial defence and active pursuit of females, the adoption of which is flexible and may be frequency dependent.

Density can also affect female reproductive success: Spence & Smith (2005, paper D) showed that in their study mean *per capita* egg production decreased at higher densities. A parentage analysis based on the same experiment (Spence *et al.* 2006b, paper E) indicated that this effect was due to females spawning smaller clutches, rather than some females being excluded from spawning. There are several possible explanations for reduced female egg production at high densities: increasing male-male aggression may interfere with female oviposition attempts; competition may arise among females for access to spawning sites; alternatively, reduced female egg production may arise through pheromonally mediated reproductive suppression. Females exposed to the pheromones of other females for several days prior to spawning have been shown to be significantly less likely to spawn compared to isolated females (Gerlach 2006). Further, dominant females produce more eggs than subordinates (Gerlach 2006). This observation is consistent with Bloom & Perlmutter's (1977) finding that females were repelled by high concentrations of female holding water. Thus, competition among both males and females may play a role in the zebrafish mating system.

Mate choice

The existence and nature of female mating preferences can be difficult to demonstrate in species where male competition plays a significant part in the mating system; matings are likely to be determined by the dominant male excluding other males rather than females actively choosing mates. There is some evidence that female zebrafish prefer larger males (Pyron 2003), and body size tends to correlate with dominance in fish (Wootton 1998). When female egg production is used as a measure of preference, female zebrafish do appear to prefer some males over others (Spence & Smith 2006, paper F). However, these preferences do not correlate with male dominance, neither do females correspond in their choice of males (Spence & Smith 2006, paper F). In view of the role played by pheromones in the reproductive behaviour of both sexes, it is possible that mating preferences may be based on olfactory cues. For instance, female zebrafish prefer the odour of unrelated males to unfamiliar brothers (Gerlach & Lysiak 2006). In the zebrafish mating system the two mechanisms of sexual selection, male-male competition and female preference, may operate in opposition. If females do not prefer dominant males, their preferences may undermine the ability of dominant males to monopolise matings. Further, competition among males for mating opportunities may be balanced by similar competition among females (Gerlach 2006). Indeed, variance in reproductive success among females is equivalent to that among males, and consequently the opportunity for sexual selection is weak in zebrafish (Spence et al. 2006b), borne out by the fact that they do not display striking sexual dimorphism.

Ovipostion choice

Females are choosy with respect to sites for oviposition. In choice tests conducted both with domesticated fish in the laboratory and with wild fish in a field-based mesocosm, females preferred a gravel substrate to silt (Spence *et al.*, paper G). Territorial males were also observed to defend gravel-substrate spawning sites in preference to silt. This preference appears to relate to spawning site quality; egg survival is enhanced by incubation in a substrate that allows oxygenated water to circulate while protecting them from disturbance and cannibalism. In the types of habitat where zebrafish are common, such as floodplain ponds, the substrate is often silty. Thus, there may be competition for access to sites that afford better water circulation as well as protection for eggs and larvae. Choice of oviposition site is one of the few ways in which oviparous species with no parental care can maximize offspring survival. Thus, if females actively choose oviposition sites, males may increase their reproductive success by guarding such sites.

SOCIAL BEHAVIOUR

Shoaling preferences

Zebrafish are a shoaling species, a behaviour that appears to be innate; fish reared in isolation quickly form shoals when placed together (Kerr 1963). McCann & Matthews (1974) showed that zebrafish reared in isolation did not discriminate between shoals of conspecifics, pearl danios, *Danio albolineatus*, or guppies, *Poecilia reticulata,* suggesting that species identification is learned. McCann & Carlson (1982) tested this by cross-rearing zebrafish with the closely related unstriped pearl danio. When fish were later allowed to choose the colour pattern of other fish with which to associate, they preferred the colour pattern of those with which they had been raised. Engeszer *et al.* (2004) replicated McCann & Carlson's results using the stripeless zebrafish pigment mutant *nacre*. Stripes appear to be a key shoaling cue; the more subtle distinction between wild-type zebrafish and leopard danios did not elicit a shoaling preference (Rosenthal & Ryan 2005; Spence & Smith in press, paper H). These studies suggest that species recognition in the zebrafish is mediated by a process of phenotype matching against a template based on early experience.

Zebrafish have also been shown to use olfactory cues in both species and kin recognition (Gerlach & Lysiak 2006). In a series of odour flume choice tests, juvenile zebrafish preferred conspecifics to heterospecifics, unfamiliar kin to non-kin, and familiar to unfamiliar kin. Thus, social preferences in zebrafish may be based on individual recognition as well as phenotype matching. Individual recognition may play a role in zebrafish since this species is known to establish dominance hierarchies (Grant & Kramer 1992; Gerlach 2006; Spence & Smith 2006, paper F). The mechanism underlying this olfactory recognition is not yet known.

Shoaling decisions in zebrafish are also influenced by shoal size and activity level. In a test of shoaling preferences, Pritchard *et al.* (2001) showed that individuals generally preferred larger shoals. However, when shoal activity level was manipulated by changing the water temperature, fish preferred the more active shoal, regardless of size. Preferences also appear to differ between the sexes (Rhul & McRobert 2005). Male zebrafish preferred to associate with female shoals compared to males but had no preference for shoal size. However, females preferred to associate with the larger shoal, regardless of whether it was composed of males or females. Zebrafish appear to be able to assess the nutritional state of conspecifics; food-deprived individuals preferred to shoal with well-fed conspecifics, and had increased foraging success than when shoaling with other food-deprived individuals (Krause *et al.* 1999).

Tests of shoaling preference have been conducted between wild-type zebrafish and various aquarium variants: leopard danios (Spence & Smith in press, paper H), longfin (Kitevski & Pyron 2003) and the transgenic Glofish^{TM}, which are genetically engineered to express red fluorescent proteins (Snesker *et al.* 2006). No significant preference was detected in any of these tests.

Aggression and dominance

Zebrafish of both sexes can establish dominance hierarchies. Aggressive interactions involve chasing and in some cases biting. Intrasexual display behaviour involves pairs of fish orienting head to tail with their fins splayed and slowly circling one another while ascending (R. Spence, pers. obs.). This behaviour operates within and between the sexes; its function is not clear but it may be a means of individual recognition which reinforces dominance ranks. Once dominance relationships become established, aggression becomes less intense (Larson *et al.* 2006). When fish are housed in pairs, the dominant individual often appears darker and utilises the entire aquarium, while subordinates are pale and occupy a smaller area in the corner (Larson *et al.* 2006). Dominance relationships appear to be relatively stable over time, at least over the duration of five-day experiments (Grant & Kramer 1992; Spence & Smith 2005, paper D). Moreover, males separated for four days have been shown to re-establish identical dominance ranks once reunited (G. Gerlach, unpublished data).

The sex of an individual does not appear to be an important factor in determining its dominance rank (Grant & Kramer 1992). The relationship between body size and dominance is unclear, partly because studies often control for size (Grant & Kramer 1992; Spence & Smith 2005, paper D, 2006, paper F). However, of studies where fish were selected to differ in size, Hamilton & Dill (2002) found that size correlated positively with rank, while Basquill & Grant (1998) found that it was not. Dominance has been demonstrated both during mating behaviour, where males establish territories around spawning sites (Spence & Smith 2005, paper D) and foraging, where dominant individuals attempt to monopolise a food source (Grant & Kramer 1992; Basquill & Grant 1998; Hamilton & Dill 2002). It is not known whether males that are territorial during spawning are also dominant during foraging.

Gillis & Kramer (1987) manipulated fish density and food patch profitability. Zebrafish formed shoals but aggressive interactions took place near feeding sites. The distribution of fish was affected by patch profitability, with more fish being concentrated around the most profitable patch. However the variability in the distribution between the three patches was greater when the density of fish was lower. At high densities there were more fish in the least profitable patch and fewer in the most profitable patch than would be predicted by an Ideal Free Distribution model (Fretwell & Lucas 1970). Aggressive interference did not fully explain the density-related reduction in foraging efficiency; aggressive interactions increased with patch profitability but decreased at high population densities. Thus, foraging distributions may also be influenced by non-aggressive interactions, while aggressive interactions are ameliorated at high densities.

Grant & Kramer (1992) used zebrafish to investigate how the temporal distribution of resources influences how individuals compete. Where resources arrive synchronously there is less opportunity for a few dominant individuals to monopolise them (Emlen & Oring 1977). If resources arrive sequentially, differences in competitive ability will generate variation in resource gain. An equivalent amount of food was delivered through a feeding tube at five different rates, varying from 3-300 min. Resource monopolisation by dominant fish increased as duration of delivery increased. Subordinate fish never displayed aggression and fed only when undetected by the dominant fish. An alternative tactic was also observed whereby some satellite fish defended the bottom of the transparent delivery tube and could thus detect the arrival of prey before the dominant fish.

Aggression and food monopolisation are also influenced by habitat. Basquill & Grant (1998) compared levels of aggression in a vegetated *versus* a non-vegetated habitat. Aggression and food monopolisation by the dominant fish were lower in the vegetated habitat. This effect could be because structural complexity reduces the effectiveness of aggression as a means of competition, or because perceived predation risk is higher in an unvegetated habitat and dominant fish are prepared to take greater risks in order to feed. In order to test these two hypotheses Hamilton & Dill (2002) compared aggression and resource monopolisation among three habitats, open, vegetated, and unvegetated with overhead cover. When allowed to choose, fish preferred to forage in the covered habitat and there was no effect of vegetation. There was no difference in aggression among habitats, but resource monopolisation was greater in the open "risky" habitat. This result suggests either that dominant fish were more willing to forage in risky habitats or that the perceived benefit of shoaling in a risky habitat outweighed the reduced foraging efficiency for subordinate fish.

Rearing environment may also influence aggression and dominance. Marks *et al.* (1999) found that fish raised in an hypoxic environment were less aggressive and spent more time in refugia than those reared in a normoxic environment. This result suggests that zebrafish offer an extremely amenable model for exploring phenotypic plasticity, particularly developmental plasticity.

Anti-predator behaviour

Increased shoal cohesion as a means of predator defence can increase vigilance and thereby reduce individual risk (Pitcher & Parrish 1993). A related behaviour is predator inspection, whereby individual fish leave a shoal briefly to approach a predator. These two traits are known to be at least partly genetically determined in zebrafish. Wright *et al.* (2003) showed differences in "boldness" (defined as propensity to inspect a novel object, in the shape of a black cylinder suspended in an experimental aquarium) among laboratory raised wild (F_2) zebrafish from four different populations. An intra-population study indicated a genetic component to shoaling tendency (the time an individual fish spent associating with a stimulus shoal), although there was no equivalent interpopulation difference. In a further study, Wright *et al.* (2006a) compared boldness and shoaling tendency between wild (F_2) and laboratory zebrafish (AB line). The AB fish showed reduced shoaling tendency and boldness compared to wild fish, presumably as a result of relaxed selection for anti-predator behaviours.

Robison & Rowland (2005) similarly compared the Nadia wild (F_5) strain with a transgenic line TMI, which contains a green fluorescent protein transgene, allowing them to be visually distinguished from other strains in a mixed aquarium. They found that Nadia were less surface orientated, were more likely to freeze on the bottom of the aquarium when presented with a novel object, and were less likely to inspect novel objects compared to TMI fish. Hybrids between the two strains showed intermediate responses and the interstrain differences were still apparent among strains reared in mixed tanks, suggesting that the behaviour was not learned. This finding with respect to inspection behaviour contradicts that of Wright *et al.* (2006a). It is difficult to make clear predictions about the effects of domestication on exploratory behaviour: there are no obvious benefits to inspection of novel objects in captivity, but neither are there costs in terms of loss of foraging time. However, the behaviours of the Nadia strain taken together might reflect a lower alarm response threshold compared to laboratory strains, which is consistent with the prediction of relaxed selection for anti-predator behaviour in laboratory strains. Freezing on the substrate may be a response among fish at risk from aerial predators (Hamilton & Dill 2002), while surface orientation is a common response of domesticated fish that are habitually fed at the surface (Robison & Rowland 2005). It is also possible that the results reflect pre-existing strain differences and are not related to domestication.

LEARNING AND RECEIVER BIAS

Learning mediates many aspects of animal behaviour, including social interactions, foraging, navigation and predator avoidance. In zebrafish, the preference for associating with other fish is innate while the preference for particular colour patterns is based on learned behaviour.

Individuals raised in isolation do not display colour pattern preferences whereas cross-reared individuals prefer to associate with the colour pattern they were raised with (Engeszer *et al.* 2004; Spence & Smith in press, paper H). Moreover, the preference effect of cross-rearing does not persist once fish are housed in groups of the same colour pattern, so the early learned preference can be modified by later experience (McCann & Carlson 1982). Learned preferences are mediated by olfactory as well as visual cues; zebrafish can differentiate between familiar and unfamiliar conspecifics on the basis of odour and thus are capable of individual recognition (Gerlach & Lysiak 2006).

The response shown by zebrafish to alarm substance is also innate but appears to function as a means of learned predator recognition. Alarm substance can initiate a conditioned response to an innocuous odour, such as morpholine, when the two are presented simultaneously (Suboski *et al.* 1990). Hall & Suboski (1995a) further elicited a learned response to a visual cue by pairing alarm substance with a red light as well as with morpholine. Thus, conditioning can operate across different sensory modalities. Hall & Suboski (1995b) also demonstrated second order conditioning whereby fish conditioned with alarm substance to respond to either light or morpholine, then learned to react to the second neutral stimulus when presented in combination with the first conditioned stimulus in the absence of alarm substance. The mechanism for communicating learned predator recognition appears to be classical conditioning, pairing of an unconditioned stimulus (alarm substance) with a conditioned stimulus (light or morpholine) to produce a conditioned response (alarm reaction). Conditioned responses can develop after a single trial, and a response can be obtained even when there is a time delay of several minutes between presentation of the unconditioned and conditioned stimuli (Korpi & Wisenden 2001). Further, conditioned responses can be passed on to naïve fish, a process known as social facilitation. Naïve fish exposed to morpholine when in the company of morpholine sensitized fish subsequently display an alarm reaction to morpholine. The naïve fish retain this learned response when solitary or in the company of a new group of naïve fish (Suboski *et al.* 1990).

An alternative approach to studying learning is to use an operant conditioning paradigm, whereby fish are trained to swim in a specific direction for a food reward paired with a visual cue. This approach has been used to study spatial memory, landmark use and orientation in other species (Braithwaite 1998), and the few studies available indicate that zebrafish are potentially a useful model for research in this area. In a study to investigate spatial learning and memory, Williams *et al.* (2002) trained adult zebrafish to swim alternately to one or other side of a divided aquarium to receive a food reward. Once trained, the fish could remember the task after a 10-day period during which they were fed *ad libitum* in another aquarium. Zebrafish were also able to learn to swim into one of three compartments when the one containing the reward was cued by a white light (Bilotta *et al.* 2005). A three-choice design provides better evidence of learning than a two-choice design as the level of a chance response is reduced to a third. Williams *et al.* (2002) reported that fish learned the task in approximately 14 trials, although Bilotta *et al.* (2005) reported wide individual variability in speed of learning. When food rewards were withheld, the training effect was quickly lost (Williams *et al.* 2002; Colwill *et al.* 2005).

Little is known about the development of learning capacity. Williams *et al.* (2002) found that age affected acquisition of conditioned responses in zebrafish. Juveniles of 6-8 weeks learned the task as well or better than adults, whereas those of 3-4 weeks were not able to do so. It was not clear whether this was a result of limited cognitive capacity or because the task presented to fish was too physically demanding. A related question, which has not been investigated in zebrafish, is the extent to which habitat complexity during rearing influences cognitive development. Research with other fishes and comparisons among populations suggests that learning in fish may be related to the demands of their environment (Odling Smee & Braithwaite 2003).

The majority of studies of learning involve testing individual fish. However, in a shoaling species like zebrafish, fish may perform better in groups; the stress of being isolated may inhibit learning ability in isolated individuals. Moreover, fish are known to be able to learn by watching others (Brown & Laland 2003). However, Gleason *et al.* (1977) found that while zebrafish learned an avoidance response to an electric shock fastest in groups of five or more, single fish learned faster than pairs. Thus the relationship between learning and group size may not be straightforward. Steele *et al.* (1991) obtained similar results in exploratory feeding behaviour in response to alanine, a ubiquitous amino acid in the aquatic environment that functions as a chemical attractant and is the primary constituent of many prey odours. They found that the fastest response was elicited in groups of four fish, but single fish responded faster than groups of two, six or eight. Group size has not been studied in relation to spatial learning in zebrafish.

Miklósi & Andrew (1999) used beads of different colours and patterns to study the effects of habituation to stimuli. Based on video footage of zebrafish biting responses they concluded that habituation is mediated by cerebral lateralisation of function; responses are controlled by different cerebral hemispheres under different circumstances. In trials, fish initially approached the bead with the right eye but in subsequent trials, once the object was familiar, used their left eye. Miklósi & Andrew concluded that right hemisphere control (i.e. left eye) mediates escape/attack responses (automatic behaviour), whereas left hemisphere (right eye) control is used in assessing novel stimuli and involves the inhibition of Mauthner cell discharge.

Many studies of learning are based on the use of neutral stimuli. However, in many species, innate receiver biases have evolved that cause them to respond more strongly to certain stimuli, and thus affect learning outcomes. Biases can exist at any level along the signal reception and processing continuum from stimulation of a primary sensory receptor to synthesis at higher levels of integration, including learning, memory and decision making (Basolo 2000). Both learned preferences and innate receiver biases operate in the context of foraging. Spence & Smith (paper I) raised groups of fish on diets consisting solely of one colour: red, blue, green or white. When fish were subsequently tested for their colour preferences in a foraging context, each group responded most strongly to red, irrespective of the colour of food with which they had been conditioned. However, there was also a significant effect of conditioning. The observed sensory bias towards red may have evolved as a function of the nature of the transmission environment that zebrafish inhabit, in combination with an adaptive preference for carotenoid compounds in their diet (Spence & Smith, paper I).

Different tasks have been shown to elicit different preferences. Colwill *et al.* (2005) used a T-maze with different coloured arms (green *versus* purple or red *versus* blue) to assess visual discrimination learning in zebrafish. They found that while fish could be trained to swim down whichever coloured arm was associated with a food reward, they learned faster and retained the response longer when the colour associated with the reward was purple or blue than when it was green or red. Thus, not only were the stimuli not perceived as equal, but the colour preferences shown in this context differed from those in the foraging study by Spence & Smith (paper I). Similarly, two studies reached different conclusions about whether zebrafish prefer a dark or light environment. Serra *et al.* (1999) found that zebrafish spent more time in a black chamber than a white one and concluded that they have an innate preference for dark environments. In contrast, Gerlai *et al.* (2000) concluded that zebrafish did not prefer a dark environment; fish initially avoided a dark chamber and on habituation spent equal amounts of time in illuminated and dark chambers. Clearly, the existence of innate preferences needs to be understood when designing behavioural protocols for learning studies in zebrafish.

GENETIC BASIS OF BEHAVIOUR

The relationship between genes and complex behaviour is not straightforward (Sokolowski 2001). Behavioural syndromes comprise numerous individual components, involving multiple, interacting genes whose expression is influenced by the environment. The starting point in such research is to identify behavioural syndromes that can be quantified, with simple, reliable protocols that allow high throughput screening, either for mutagenesis or naturally occurring behavioural variation. Much of the pioneering behavioural genetics research has utilised the fruitfly as a model. In this species, genes have been identified that control complex syndromes such as learning and memory, mating behaviour and circadian rhythms (Sokolowlki 2001; Anholt & Mackay 2004). The advent of functional genomics has enabled research to be extended to other species (Fitzpatrick *et al.* 2005; Vasemägi & Primmer 2005).

In comparison, there is a paucity of studies on complex behaviour in zebrafish, although it is recognised as having great potential as a model for understanding the genetic basis of human behavioural disorders (Patton & Zon 2001; Guo 2004). One area of interest has been the effect of drugs of abuse on behaviour. Darland & Dowling (2001) conducted a behavioural screen for cocaine addiction using the conditioned place preference paradigm (CPP), whereby the drug is paired with a neutral stimulus in one compartment of the aquarium and the amount of time the fish spends in each compartment is measured before and after administration of the drug. Three families showed abnormal responses in the CPP and were subjected to further behavioural screens, testing spatial cognition in a T-maze, swimming behaviour, and sensitivity to light. Each family had different behavioural profiles, which were shown to be heritable, each supposed as representing a different single gene mutation that affected addiction (Darland & Dowling 2001).

Gerlai *et al.* (2000) designed a series of simple, easily quantifiable tests to examine the effects of alcohol administered at different concentrations on locomotion, aggression, shoaling tendency, alarm response, light/dark preference and pigmentation. These tests could be used to identify individuals with abnormal responses to alcohol. Dlugos & Rabin (2003) compared the effects of acute and chronic alcohol administration on three zebrafish strains. The strains differed in startle response and shoal cohesion, suggesting that there is a genetic basis to both initial sensitivity and the development of tolerance to alcohol.

Zebrafish may also be a suitable model for studying the genetic basis of social behaviour. Larson *et al.* (2006) showed that there are clear differences between dominant and subordinate fish in the expression of arginine vasotocin, a neurohormone known to mediate social behaviour such as aggression, courtship and parental behaviour in vertebrates, although the system varies among taxa. Dominance relationships are not fixed and must, therefore, involve differential expression of different genetic pathways.

Tropepe & Sive (2003) suggested that a forward genetics screening approach might be employed to model the behavioural deficits involved in autism using zebrafish. As deficits in social behaviour are strongly characteristic of autism, behaviours such as courtship and shoaling may represent a suitable paradigm for sociability. In mice, tests of exploratory behaviour have been used as a paradigm for anxiety and fear, exploratory behaviour tending to be negatively correlated with anxiety (Flint *et al.* 1995). Using a similar approach, Wright *et al.* (2006a) utilised the pronounced differences between wild and laboratory strains of zebrafish in willingness to approach an unfamiliar object (boldness) and attempted to identify quantitative trait loci associated with these.

Other complex behaviours which offer potential for genetic analysis are learning and memory. Appetitive choice protocols can be used to assess speed of learning and retention time between different strains of fish, fish reared under different conditions, or known behavioural mutants. Genetic analysis of cerebral lateralisation of function may offer insights into the molecular basis of habituation. For instance, the mutant *frequent situs inversus* (*fsi*), which shows reversal of asymmetry in many cerebral and visceral organs, showed reversal of behavioural asymmetry in some tests but not others, suggesting that at least two different mechanisms are involved in lateralisation of function (Barth *et al.* 2005).

CHAPTER TWO: ECOLOGY

Paper B: The distribution and habitat preferences of the zebrafish in Bangladesh

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Authors: Rowena Spence, M. Kaniz Fatema, Martin Reichard, K. Anisul Huq, Md. Abdul Wahab, Zoarder F. Ahmed and Carl Smith.

Abstract

The present study presents the results of a survey of a wide range of water bodies in Bangladesh to identify and describe zebrafish habitat preferences. Field-based experiments were conducted to determine the vertical distribution of zebrafish in the water column, together with five other fish species commonly found in association with zebrafish. The zebrafish is a floodplain rather than riverine species, being most abundant in shallow lakes, ponds and ditches, typically in open locations with relatively clear water and abundant vegetation at the margins. It is commonly found in water bodies with a connection to rice cultivation and is more common in the north of Bangladesh than the south. The zebrafish occupies the whole of the water column and is observed as frequently in open water as amongst aquatic vegetation.

INTRODUCTION

The purpose of this study was to identify and describe the habitat preferences of the zebrafish, assess its relative abundance within local fish assemblages, and observe its interactions with other fish species as a precursor to more detailed research into its natural ecology and behaviour.

The natural range of the zebrafish is centred around the Ganges and Brahmaputra river basins in north-eastern India, Bangladesh, Nepal and northern Myanmar (Barman 1991), though it has also been reported from southern India (collection of the Swedish Natural History Museum), and Jammu in Kashmir (Dutta 1993). Although wild zebrafish have been collected previously for taxonomic (Pritchard 2001; http://artedi.nrm.se/nrmfish) and population genetic analysis (Gratton *et al.* 2004; Wright 2004) no systematic attempt has been made to describe its natural ecology, other than brief descriptions in fish identification guides. The zebrafish is typically described as inhabiting slow-moving or standing water bodies, particularly rice-fields (Sterba 1962; Talwar & Jhingran 1991; Jayaram 1999), although it is also reported as inhabiting rivers and hill streams (Daniels 2002). The present study concentrated on its habitat preferences in the Ganges and Brahmaputra drainages in Bangladesh.

Bangladesh has a monsoon climate with wide seasonal variation in the extent of freshwater habitats. Even in the dry season there is a wide range of aquatic environments. The main river systems, the Ganges and Brahmaputra, rise in the Himalayas, and run through low-lying areas known as "hoars", which flood extensively during the monsoon months from June-September. Rivers in this region continually change their course over the floodplain, leaving networks of oxbow lakes and blind channels or "beels", which may have seasonal connections to the main river. In addition, the country has extensive areas of man-made lakes, ponds and irrigation channels, constructed for fish and rice cultivation. The present study involved sampling across the full range of these habitats. In addition, an experiment was conducted to observe its vertical distribution in the water column, under semi-natural conditions, together with five other fish species with which it was commonly found.

METHODS

General distribution

Sample sites

A survey was conducted during the dry season, in January 2005. This month was chosen to facilitate access to a wide range of sites and identify discrete populations. Surveys were conducted in two areas, Khulna District in the south of Bangladesh in the River Ganges drainage (GPS reading 22°N, 90°E), and Mymensingh District, to the north, in the River Brahmaputra drainage (GPS reading 24°N, 90°E), see distribution map on page 12. A total of 23 sites were sampled in January 2005. In addition, a further three sites in Mymensingh District were sampled in August 2005 during the monsoon season. Sites sampled in August were temporary habitats, connected to inundated areas and representing additional habitat types unique to the monsoon season. A description of all study sites is summarised in Table I.

Sampling procedure

Site position was recorded using a GPS handset. Sampling was conducted using a fine mesh seine net (5.0 m x 1.3 m), with mesh size varying from 2-7 mm. Between three and five hauls were taken over a distance of 5 m at each site and all fishes captured were retained live. A representative subsample of the fishes caught at each site was retained and fixed in 4% formalin. Specimens of any newly encountered species were also preserved in 4% formalin. All species collected were identified (Sterba 1962; Talwar & Jhingran 1991; Roberts 1994, 1998; Jayaram 1999; Daniels 2002) and specimens in subsamples were counted and their SL measured to the nearest mm.

Table I. List of sampling sites showing location, basic site description and approximate size of the area in which sampling was conducted.

Site	Location	Description	Approx. area (m)
	Khulna District:		
1	Khulna University Campus	Ditch surrounding a series of ponds	3 x 90
2	Bagerhat	Large artificial lake	600 x 600
3	Khulna University Campus	Seasonally isolated arm of a canal	10 x 500
4	Khulna University Campus	Small pond treated with lime and rotenone	8 x 10
5	Khulna University Campus	Semi-natural pond	10 x 15
6	Khulna University Campus	Natural ponds	4 x 8
7	Golamari	Village pond near Golamari Bridge	25 x 50
8	Bhatiaghata River	A tributary of the Ganges	150 (width)
9	Golamari	Isolated channel of the Golamari River	200 x 1500
10	Khulna University Campus	Large artificial pond	15 x 30
11	Golamari	Isolated channel adjacent to site 9	100 (width)
12	Golamari	Established irrigation channel	10 (width)
13	Golamari	Isolated channel	100 (width)
	Mymensingh District:		
14	Sutiakhali	Pond used for pearl mussel culture	17 x 20
15	Sutiakhali	Shallow cultivated pond connected to rice-field.	30 x 50
16	Sutiakhali	Isolated pond	10 x 12
17	Sutiakhali	Isolated pond	10 x 10
18	Sutiakhali	Isolated pond	10 x 12
19	Brahmaputra River	An old arm of the Brahmaputra River	150 (width)
20	Dhuno River, Balikhala	A large river in a low-lying ‘hoar’ region	200 (width)
21	Haibatnagar	Old fish farm	30 x 30
22	Mymensingh University Campus	Semi-natural pond	150 x 150
23	Mymensingh University Campus	Ditch connecting to rice-fields	10 (width)
	Further sites sampled in August:		
24	Mymensingh University Campus	Small semi-natural pond	8 x 15
25	Mymensingh University Campus	Channel adjacent to and feeding into campus	8 (width)
26	Brahmaputra River	A creek flowing out from the main river channel into extensive flooded area	15 (width)

At each site the following environmental variables were recorded: geographic location, connection with other water bodies, dimensions, mean depth, Secchi depth, temperature, pH, ammonia/ammonium, salinity, and substratum type. Specimens of aquatic vegetation were collected, pressed and later identified using the botanical collection in the herbarium of the University of Leicester.

Vertical distribution

An experiment was conducted at the Fisheries Field Laboratory of the Faculty of Fisheries at the Bangladesh Agricultural University, Mymensingh in August 2005. Experiments were conducted during the monsoon season, which corresponds with the zebrafish spawning season (Daniels 2002; Spence *et al.* in press, paper C). Experiments were performed in glass-sided outdoor aquaria (246 x 30 x 84 cm) filled with pond water to a depth of 60 cm. Environmental conditions (light, temperature, rainfall and water quality) were identical to those in the surrounding ponds from which experimental fish were collected, (sample sites 22, 23, 24 and 25). Water temperature ranged from 29-33 °C. During mid-afternoon, rice-straw screens (108 x 208 cm) were placed on top of the aquaria to provide shade.

Four species that were shown from spatial distribution surveys to commonly occur with zebrafish were used in the experiment; blue panchax (*Aplocheilus panchax*), flying barb (*E. danricus*), highfin glassy perchlet (*Parambassis lala*), and dwarf gourami (*Colisa lalia*). In addition, juvenile Nile tilapia (*Oreochromis niloticus*), a widely introduced species in Bangladesh, were used. All fishes were collected from among the same group of ponds and were randomly assigned to test aquaria, with no specific sex ratio being used. Seven replicates were conducted over two days. Each aquarium was stocked with 10 zebrafish, five blue panchax, six flying barbs, five highfin glassy perchlets, three dwarf gouramis and five Nile tilapia, the numbers of fishes being chosen to match the mean relative abundance of each species from previous surveys. The mean ± S.D. SL (mm) of fish used in the experiment were as follows: zebrafish, 24.4 ± 3.58; blue panchax, 29.4 ± 5.36; dwarf gourami, 34.1 ± 4.29; highfin glassy perchlet, 21.3 ± 2.55; flying barb, 34.5 ± 5.11; Nile tilapia, 28.4 ± 6.05.

Two bundles of aquatic plants were placed in one half of each aquarium and four plastic boxes (150 x 100 x 40 mm), filled with gravel, were placed on the bottom, approximately 50 cm apart. Wild and domesticated zebrafish readily use boxes of gravel for oviposition; the eggs fall between the gravel, which prevents egg cannibalism. Three vertical sections (top, middle, bottom) were marked on each aquarium with a horizontal line at 20 cm and 40 cm. Fishes were fed three times each day with zooplankton collected from adjacent ponds, together with commercial dry food.

Behavioural observations of fishes in each aquarium were conducted twice; at dawn (approximately 0530 AM) when zebrafish spawn, and after spawning between 1100 AM and 1200 noon. During each period of observation the position in the aquarium of every individual of each species was recorded five times. There were six possible positions: top, vegetated; top, open; middle, vegetated; middle, open; bottom, vegetated; bottom, open. The total duration of each observation period was 15 min, the position of every individual of each species being recorded every 3 min. In addition, any aggressive interactions among species and feeding in spawning boxes were recorded. After the second observation period, the spawning boxes were checked for the presence of zebrafish eggs. At the end of the experiment, all fishes were removed, their SL measured to the nearest mm, and returned to the ponds from which they had been collected.

Data analysis

All data were tested for normality using a Kolmogorov-Smirnov test and for equality of variance using a Bartlett's test. A principal component analysis (PCA) was used to identify relationships among the following variables at each sample site: locality; flow; depth; Secchi depth; substratum; presence of vegetation; salinity; pH; ammonia/ammonium; presence of predators; connection with rice cultivation. The correlation between each of the first two principal components and zebrafish $\log_{10}$ abundance was then examined.

Paired *t*-tests were used to compare the distribution of each species between the two observation periods in the vertical distribution experiment. A log-linear contingency table (Everitt 1977) was used to test for the effects of height in the water column and vegetation cover on the distribution of each species in aquaria. In all but one species there was no significant difference in distribution between the two time periods so a two-way contingency table was used. In the case of the remaining species, a three-way contingency table was used, taking account of time period in addition to height and vegetation cover. The analysis of each species separately made the untested assumption that their distribution was independent of the rest of the assemblage. However, the aim was to test whether apparent patterns of distribution of each species were statistically significant and no inferences were made about the basis for the observed distributions.

Hurlbert's measure of niche breadth was also calculated for each species in the vertical distribution experiment. This is a measure of the uniformity of distribution of individuals among resource states or habitats and is given by the equation:

$$B' = \frac{1}{\sum (p_j^2 / a_j)}$$

where p = proportion of individuals, j = resource states and a = available resources. Thus, the smaller the number, the narrower the niche breadth and the more specialised the species.

RESULTS

General distribution

Zebrafish were found in nine out of the 26 sites sampled, two in Khulna District and seven in Mymensingh District. The environmental variables recorded for each sampling site, together with $\log_{10}$ abundance of zebrafish, are shown in Table II. Zebrafish were more abundant in the north (Mymensingh) and were not found in any of the rivers sampled. This result was reflected in the PCA. The first two principal components accounted for 32% and 18% of the variance respectively (Fig. 1). The first component (PC1, loadings in parentheses) related to depth (-.47), flow (-.46), substrate (-.46), transparency (-.42) and the presence of predators (-.39). The second component (PC2) related to a connection to water bodies associated with rice cultivation (-.58) the presence of vegetation (-.48) and locality (-.34). There was no correlation between PC1 and the occurrence of zebrafish across all sampling sites (Pearson correlation: $r_p = 0.29$, $N = 26$, $P = 0.144$). However, in the nine sites where zebrafish were found, PC1 significantly correlated with zebrafish $\log_{10}$ abundance (Pearson correlation: $r_p = 0.85$, $N = 9$, $P = 0.004$). PC2 was negatively correlated with the $\log_{10}$ abundance of zebrafish across all sampling sites, (Pearson correlation: $r_p = -0.46$, $N = 26$, $P = 0.017$).

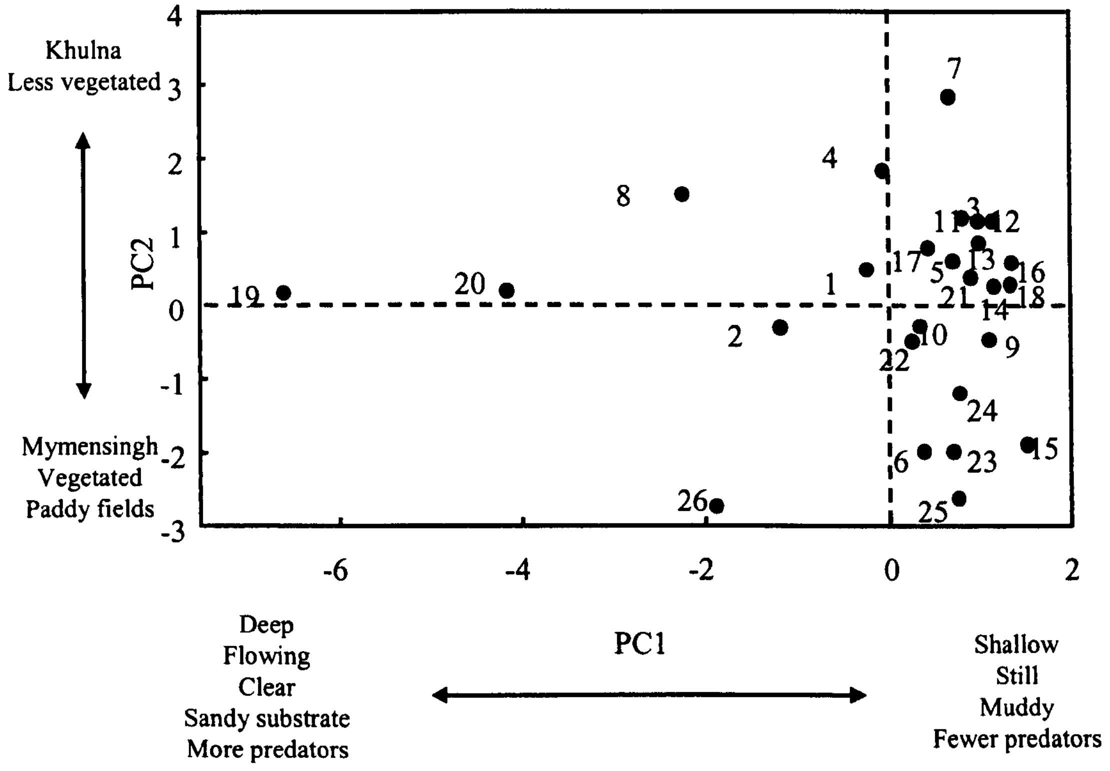

Figure 1: Scores for each sampling site (see Table II) on the two principle components

Table II. Environmental variables for each sampling site: **Khulna District:** 1. Ditch; 2. Large artificial lake; 3. Seasonally isolated arm of a canal; 4. Pond treated with lime and rotenone; 5. Semi-natural pond; 6. Natural ponds; 7. Village pond; 8. Bhatiaghata River; 9. Isolated river channel; 10. Artificial pond; 11. Isolated river channel; 12. Irrigation channel; 13. Isolated river channel. **Mymensingh District:** 14. Cultivated pond; 15. Cultivated pond; 16. Isolated pond; 17. Isolated pond; 18. Isolated pond; 19. Brahmaputra River; 20. Dhuno River; 21. Old fish farm; 22. Semi-natural pond; 23. Ditch; 24. Small semi-natural pond; 25. Channel; 26. Creek.

Site	*D. rerio* $\log_{10}$ abundance	Flow	Depth (cm)	Secchi depth (cm)	Substrate	Vegetation	Temp. (^{0}C)	pH	Ammonia/ Ammonium (mg l^{-1})	Salinity	Connection with paddy	Predators
1	1	Still	80	51	Mud		20	8	0.01	0		Yes
2	0	Still	100	92	Mud	Yes	22	8	0.01	0		Yes
3	0	Still	80	16	Mud		18.5	8	0.1	0.4		
4	0	Still	73	37	Mud		20	7.4	0.1	0.2		Yes
5	0	Still	50	50	Mud	Yes	17.5	7.6	0.1	0.2		
6	0	Still	35	35	Mud	Yes	20	8	0.1	0	Yes	Yes
7	0	Still	85	37	Mud		18	7.4	0.1	0.8		
8	0	Running	125	75	Mud		21.5	8	0.1	0.6		Yes
9	1	Still	50	19	Mud	Yes	20	8	0.1	0		
10	0	Still	71	64	Mud	Yes	20.5	8	0.1	0		
11	0	Still	73	52	Mud	Yes	20.3	8	1	0.4		
12	0	Still	50	44	Mud		19	8	0.1	0.4		
13	0	Still	76	23	Mud		22	8	0.1	0.2		
14	0	Still	30	16	Mud	Yes	20	7.6	0.01	0.6		
15	3	Still	15	15	Mud	Yes	20.5	8	0.01	0.6	Yes	
16	2	Still	40	15	Mud		19.5	8	0.01	0.6		
17	2	Still	103	30	Mud		16.5	8	0.01	0.6		
18	0	Still	25	32	Mud		19	8	0.01	0.6		
19	0	Running	200	197	Sandy Mud	Yes	21	8	0.01	0.4		Yes
20	0	Running	130	46	Sandy Mud	Yes	21	7.6	0.01	0.4		Yes
21	0	Still	80	12	Mud		22	8	0.01	0.4		
22	1	Still	96	31	Mud	Yes	21	8	0.01	0.4		
23	2	Still	50	50	Mud	Yes	23	8	0.01	0.4	Yes	
24	2	Still	65	15	Mud	Yes	33	8	0.01	0		
25	2	Still	75	15	Mud	Yes	33	8	0.01	0	Yes	
26	0	Running	120	15	Mud	Yes	30	8	0.01	0	Yes	Yes

A total of 48 fish species from 16 families were captured across the 26 sites (Table III). Cyprinids were the dominant group in the assemblage, with zebrafish among the most abundant species. Highfin glassy perchlet, elongate glassy perchlet (*Chanda nama*), flying barb, blue panchax, tank goby (*Glossogobius giuris*), ricefish (*Oryzias carnaticus*), together with barbs (*Puntius* spp.) and gouramis (*Colisa* spp.), while less abundant, were more widely distributed than zebrafish. Some sites had been used for aquaculture and contained commercial species, such as bighead carp (*Aristichthys nobilisi*), catla (*Catla catla*), mrigal (*Cirrhinus cirrhosus*), silver carp (*Hypopthalmichthys molitrix*), rohu (*Labeo rohita*), and Nile tilapia. The main predatory taxa captured were snakeheads (*Channa* spp.) and freshwater garfish (*X. cancila*).

Vertical distribution

The only species to show a significant difference in its distribution between observation periods was the blue panchax (paired *t*-test, square root transformed data: $t_{42} = 19.99$, $P < 0.001$). In both time periods, there was a significant effect of height in the water column on fish distribution but not vegetation cover (Chi squared: $\chi^2_7 = 19.20$, $P = 0.008$, Fig. 2).

There was no significant difference in the distribution of the other five species between the two observation periods ($P > 0.05$). There were no significant effects of either height in the water column or vegetation cover on the distribution of zebrafish (Chi squared: $\chi^2_2 = 0.59$, $P = 0.745$). Similarly, flying barbs showed an even distribution across all sections of the aquaria (Chi squared: $\chi^2_2 = 0.49$, $P = 0.785$). For dwarf gouramis there was a significant effect of vegetation, but not of height in the water column and there was no interaction (Chi squared: $\chi^2_2 = 14.59$, $P = 0.001$). In contrast, for highfin glassy perchlets there was a significant effect of height in the water column (Chi squared: $\chi^2_2 = 9.47$, $P = 0.009$) but not of vegetation and there was no interaction. For Nile tilapia there was a significant effect of height in the water

Table III. Fish species recorded in each sampling site (see Table II)

	Khulna District													Mymensingh District												
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
AMBASSIDAE																										
Chanda nama		x	x			x	x	x	x	x		x	x									x			x	x
Parambassis lala	x		x		x	x		x	x	x		x	x			x	x				x	x	x		x	x
ANABANTIDAE																										
Anabas testudineus	x											x														
Badis badis	x	x	x			x							x									x	x			x
Channa punctatus	x	x		x								x														x
Channa orientalis																			x							x
Colisa chuna										x															x	
Colisa lalia	x	x	x		x	x			x	x	x	x	x		x	x	x					x	x		x	
Colisa fasciatus	x																									
ANGUILLIDAE																										
Anguilla bengalensis	x																									
APLOCHEILIDAE																										
Aplocheilus panchax	x	x	x		x	x			x	x	x	x	x	x	x		x					x	x	x	x	x
Oryzias carnaticus	x		x	x	x	x	x	x	x	x	x	x	x						x		x					x
BAGRIDAE																										
Mystus vittatus																			x							x
BALITORIDAE																										
Acanthocobitis botia																			x							x
Nemacheilus sp.																										x

Table III continued

	Khulna District													Mymensingh District												
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
BELONIDAE																										
Xenedonton cancila						x		x	x										x	x						
CICHLIDAE																										
Oreochromis niloticus				x											x									x		
CLUPEIDAE																										
Corica soborna									x																	
COBITIDAE																										
Botia dario																			x							x
Lepidocephalichthys menoni																										x
Neoeucirrhichthys maydelli																			x							
CYPRINIDAE																										
Amblypharyngodon mola	x		x											x		x										x
Aristichthys nobilis	x																				x					
Catla catla				x																						
Cirrhinus cirrhosus	x																									
Danio rerio	x								x						x	x	x					x	x	x	x	
Devario devario																										x
Esomus danricus	x		x	x	x	x	x		x	x	x	x	x	x	x	x	x				x	x		x	x	x
Hypophthalmichthys molitrix	x																									
Labeo rohita				x															x							x
Puntius chola	x											x			x											x
Puntius conchonius											x	x	x													x
Puntius gelius	x	x	x																		x					x
Puntius phutunio	x		x	x	x	x			x	x	x										x					x

Table III continued

	Khulna District													Mymensingh District												
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Puntius sophore			x																							x
Puntius terio	x	x							x		x															x
unidentified *Puntius* spp.						x																				x
Salmostoma phula														x			x			x						
ELEOTRIDAE																										
Eleotris sp.										x																
GOBIIDAE																										
Brachygobius nunus									x		x	x	x						x							
Glossobobius giuris	x	x	x	x	x		x			x	x	x	x	x			x		x		x					x
Gobiopterus chuno									x			x	x													
MASTACEMBALIDAE									x																	
Macrognathus sp.																										x
Macrognathus aculeatus																										x
Macrognathus pancalus																			x							x
Mastacembelus armatus																										x
NANDIDAE																										
Nandus nandus																										x
SILURIDAE																										
Wallago attu																										x

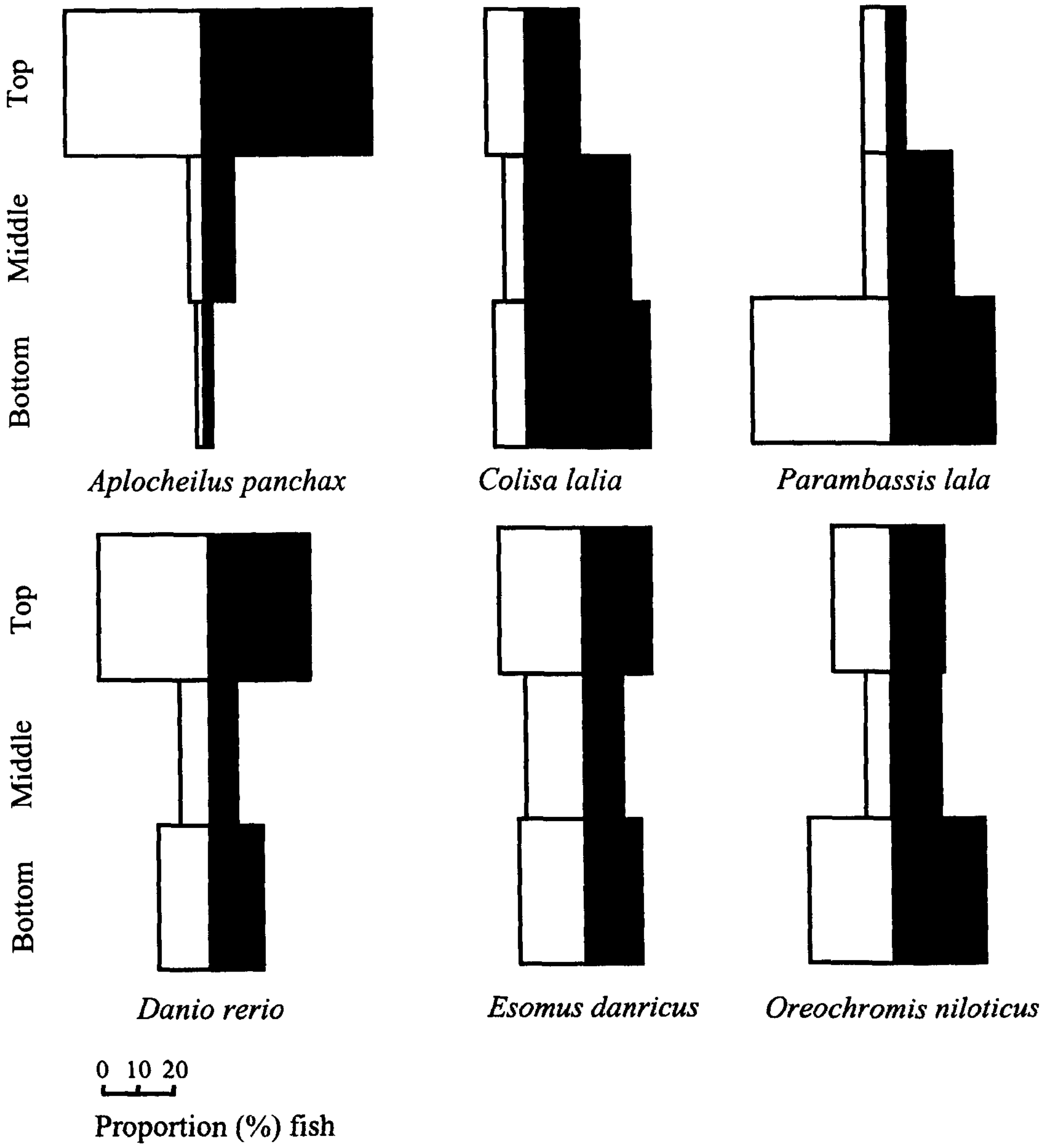

Figure 2: Mean proportion (%) of each species in: ■ vegetated and □ unvegetated sides of experimental aquaria, at different heights in the water column, averaged over two 15 minute observation periods.

column (Chi squared: $\chi^2_2 = 8.25$, $P = 0.016$) but not of vegetation cover and there was no interaction.

The Hurlbert's *B'* scores for each species, together with estimates of their 95% CL, are shown in Table IV. Of the six species, blue panchax (*A. panchax*) were the most specialised in their habitat use and flying barbs (*E. danricus*) the most generalist.

Table IV. Hurlbert's *B'* measures of niche breadth for six species, based on the proportion of each species in vegetated and unvegetated sides of experimental aquaria, at different heights in the water column.

Species	Score	95% CL
Aplocheilus panchax	0.46	0.28
Colisa lalia	0.79	0.70
Parambassis lala	0.61	0.38
Danio rerio	0.79	0.36
Esomus danricus	0.96	0.26
Oreochromis niloticus	0.86	0.45

DISCUSSION

The zebrafish was more abundant in samples from the northern than from southern areas sampled. Khulna probably represents the southern limit of its range in Bangladesh; further south is the northern fringe of the Sundarban mangrove forest, and here the water becomes increasingly saline and the zebrafish is probably absent (C. Smith, unpublished data). The zebrafish appears to be a floodplain rather than a true riverine species. Zebrafish were not found in rivers or temporary creeks that opened during the monsoon season. However, genetic analysis has shown that there is considerable mixing of populations (Gratton *et al.* 2004) and some of the sites where zebrafish were found may connect with the main river channel during the monsoon. Sample site descriptions by Fang & Roos (http://artedi.nrm.se/nrmfish) and

McClure *et al.* (2006) largely correspond with the present findings, although zebrafish were also found in streams with moderate and, in one case, fast flow (http://artedi.nrm.se/nrmfish/). When zebrafish were found in rivers, they tended to be confined to the slower-flowing areas at the margins (Mclure *et al.* 2006; D. Edds, pers. comm.).

All the sites where zebrafish were captured were shallow, relatively clear and frequently with aquatic vegetation. Predatory species were found in only one site with zebrafish (site 1), which also had the lowest abundance of zebrafish. Two predatory fish taxa were captured (snakeheads and freshwater garfish) which may feed on zebrafish. However, the sampling protocol may have failed to capture other potential fish predators such as nocturnal catfishes. Avian predators such as the Indian pond heron, *A. grayii,* and the common kingfisher, *A. atthis,* were also ubiquitous in the area and may feed on zebrafish.

All the sample sites were subject to a degree of human influence. Some of the cultivated ponds had probably been treated with lime and rotenone prior to the introduction of commercial species. Introduced species such as Nile tilapia were common in these sites. Many ponds contained commercial and introduced species as well as small indigenous species, although none of the sites sampled were the subject of active fish cultivation at the time of sampling. Every site sampled was actively exploited for fishery purposes; small indigenous species (<25 cm total length) form a substantial constituent of the protein intake of poor households in Bangladesh (Wahab 2003). The zebrafish is among the smallest of these small species and is not, therefore, considered an important food resource even by subsistence fishermen.

Although the zebrafish was relatively widespread and abundant among sampling sites, in two sites (15 and 23) it was the most abundant species. However, it was not as widespread as some of the other species in the assemblage, particularly the flying barb, another cyprinid and the sister group to *Danio* (Fang 2003). One notable feature of the results was that zebrafish tended to be abundant in habitats connected to rice cultivation at the time of sampling, though not in rice-fields themselves. There is no clear explanation for this association with rice cultivation. However, it could be related to the use of fertilizers, which may promote the growth of zooplankton on which zebrafish chiefly feed (Dutta 1993; Spence *et al.* in press, paper C).

Females collected in January from one site (site 23) were found, on dissection, to contain mature ova. This finding contradicts anecdotal evidence that the zebrafish has a discrete breeding season only during the monsoon (Daniels 2002). It may be that breeding is dependent on food availability rather than season *per se*. Zebrafish specimens ranged in size from 15 mm to 34 mm SL. It is probable that the few large specimens caught in January represent a different year class. Thus, the zebrafish is probably predominantly an annual species, breeding primarily during the monsoon, but spawning continuously after achieving maturity (Spence *et al.* in press, paper C).

The results of the experimental study of vertical distribution show that zebrafish, flying barb and Nile tilapia were generalist in their habitat use, while blue panchax, highfin glassy perchlet and dwarf gourami were more specialised (Fig. 2). Blue panchax tended to spend a greater proportion of their time near the water surface, while highfin glassy perchlet and Nile tilapia spent more time towards the bottom. This result is reflected in the scores for Hurlbert's *B'*, the zebrafish's score being in the middle of the range for the six species. The zebrafish did not spawn during the experiment although they did spawn in the same experimental aquaria in a separate experiment (Spence *et al.*, paper G). They swim close to the bottom during spawning (Spence & Smith 2005), although courtship involves males actively pursuing females, which utilise the entire water column. No aggressive interactions with other species were observed during the experiment.

In conclusion, the zebrafish was found to be relatively abundant in Bangladesh. It was most commonly found in shallow ponds and standing water bodies in open locations with aquatic vegetation in the margins. It was also frequently encountered in water bodies with a connection to rice cultivation and was more common in the samples from the north of Bangladesh than the south. The zebrafish occupied the whole of the water column and it was observed as frequently in open water as amongst aquatic vegetation.

Paper C: Diet, growth and recruitment of wild zebrafish in Bangladesh

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Authors: Rowena Spence, M. Kaniz Fatema, Shafeek Ellis, Zoarder F. Ahmed and Carl Smith

Abstract

Gut content analysis showed the diet of zebrafish in Bangladesh consists primarily of zooplankton and insects. Length-frequency analysis indicates that zebrafish are an annual species, with the main period of reproduction commencing just before the onset of the monsoon season. Growth rates vary with age and season, with the period of most rapid growth in early life during the monsoon months.

INTRODUCTION

The purpose of this study was to describe temporal changes in the diet of wild zebrafish using gut content analysis, and identify recruitment patterns and estimate growth parameters using length-frequency analysis. There are few data on the diet of wild zebrafish and none based on sampling throughout the year. There are no published studies on growth rates or recruitment patterns of zebrafish in natural conditions. On the basis of previous published studies (Dutta 1993; McLure 2006) it was predicted that diet would consist primarily of zooplankton and insects.

The floodplains of Bangladesh represent a dynamic environment with wide seasonal variation in available habitat. Food availability is also likely to be much higher during the summer monsoon months due to high temperatures together with flooding of terrestrial areas which increases the circulation of nutrients (Talling & Lemoalle 1998). Anecdotal evidence suggests that recruitment in zebrafish coincides with the monsoon (Daniels 2002). The zebrafish is small species with rapid growth and development (Eaton & Farley 1974a; Kimmel *et al.* 1995). As such, and because of the dynamic nature of the floodplain environment, it was predicted that zebrafish would be an annual species, recruitment commencing at the beginning of the monsoon season (May) and the main period of growth taking place during the summer months.

METHODS

This study was conducted at the Fisheries Field Laboratory of the Faculty of Fisheries at the Bangladesh Agricultural University, Mymensingh. The study population occupied a small lake of approximately 3.42 ha with an average depth of 1.2 m and a silt substrate and abundant aquatic vegetation at the margins. The annual range in water temperature was 17-33 °C. A sample of approximately 120 fish was collected each month from January to December

2005 using a fine mesh seine net (5.0 m x 1.3 m), with a mesh size varying from 2-7 mm. After capture, fish were immediately killed with an overdose of anaesthetic and fixed in 4% formalin. The SL of all preserved fish was measured to the nearest 0.1 mm using electronic calipers. A length-frequency analysis based on 2 mm size classes was conducted using FiSAT (Gayanilo & Pauly 1997). Gut contents were analysed from a randomly selected sub-sample of 25 fish in each monthly sample; a pilot study showed that a sample size of 25 was representative of the entire sample. The alimentary canal was removed and gut contents examined under a dissecting microscope and identified to Class or Order (Ward & Whipple 1959). The proportion by volume of different categories of food items was estimated visually.

RESULTS

Diet

The main identifiable components of the diet were zooplankton and insects, although phytoplankton, filamentous algae and vascular plant material were also found, as well as eggs, probably of cladocerans (Table I). The majority of insect remains were too fragmented or degraded to permit further identification. Insects that could be identified to order were primarily dipterans, although hemipterans, hymenopterans and coleopterans were also found. The majority of insects were aquatic species, or aquatic larval forms of terrestrial species, with dipteran larvae being particularly common during the monsoon months (June-August). However, some terrestrial insects and arachnids were also present. There was a large proportion of unidentifiable material in the samples, which included an inorganic component.

There was no significant difference in the composition of diets between male and female zebrafish, based on a comparison of the relative proportion by volume of the 14 different categories of food items (unpaired *t*-test, $\log_{10}$ transformed data: t_{26}, $P > 0.05$). The

Table I. Mean proportion (%) by volume of different categories of gut contents of zebrafish over the course of one year.

	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec	**Mean**	**SD**
Zooplankton														
Cladocera	4.4	10.5	35.8	9.5	2.8	11.4	9.7	19.6	7.5	10.4	32.8	14.8	14.7	10.8
Copepoda	0	4.0	1.9	7.7	14.8	18.6	1.6	2.5	16.4	0	8.0	0	6.3	6.8
Ostracoda	12.4	43.1	12.8	0.8	14.0	9.8	4.8	2.8	40	3.2	0.4	12.2	12.2	14.5
Insects														
Coleoptera	0.8	0	0	0	0	0	0	0	0	0	0	0.4	0.1	0.2
Diptera	1.2	1.2	0	0	0.5	0	3.4	9.2	15.2	6.4	3.7	2.1	3.6	4.6
Hemiptera	2.0	0	0	0	0	0	0	0	0	0	0	0	0.2	0.6
Hymenoptera	0.4	2.2	5.0	1.4	1.4	2.0	0	0	0.8	2.5	0	0.8	1.4	1.4
Miscellaneous	22.8	7.9	18.2	18.2	12.0	17.4	28.4	21.4	27.6	30.0	16.4	20.8	20.1	6.6
Others														
Algae	0	9.5	4.4	13.3	8.0	3.0	0	0.2	1.6	0	7.6	0	4.0	4.6
Arachnida	2.8	2.1	0	0	0	0	0.8	0.2	0	0	0	0	0.5	1.0
Invertebrate eggs	0	4.1	5.3	6.3	2.2	0.6	3.2	1.4	0.4	0	0.8	0	2.0	2.2
Phytoplankton	0	0	0	0	0	0.1	1.2	0.1	0	0	0	0.4	0.2	0.4
Vascular plants	0	0	0.4	3.6	0.8	5.2	6.0	1.5	0.4	0	2.0	0	1.7	2.1
Unidentifiable	53.2	16.7	16.2	38.7	44.0	28.5	21.2	45.0	24.0	16.3	49.5	43.5	33.0	14.0

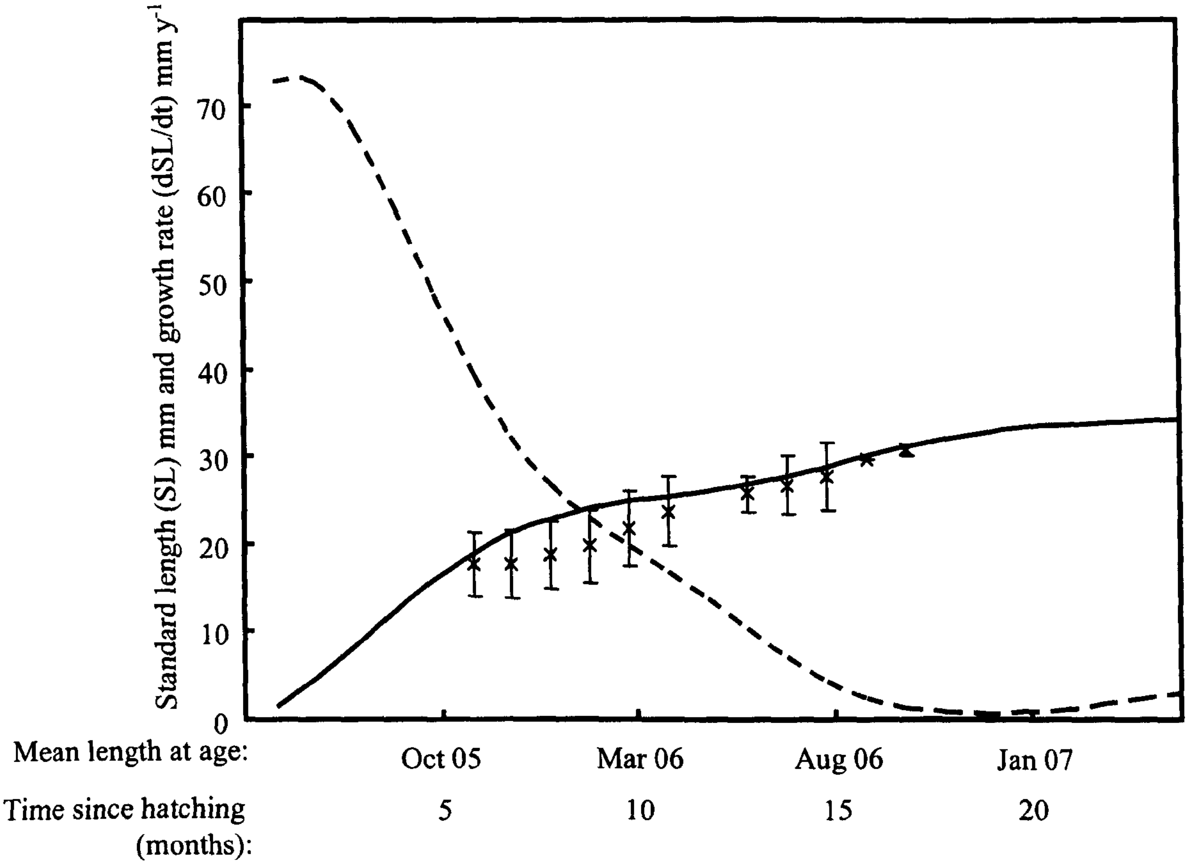

Figure 1. Change in SL (solid line) and growth rate (broken line) as a function of time (months) since hatching, modelled using the seasonalized Von Bertalanffy growth equation. Crosses denote observed mean length at age of fish with a putative spawning date of 1 June 2005, ± SD.

proportions of zooplankton and insects differed significantly among months (one-way ANOVA, ranked data: zooplankton $F_{11,288}$, $P < 0.001$; insects $F_{11,288}$, $P < 0.01$), although there was no clear seasonal pattern (Table I).

Growth and recruitment

Two distinct size classes of zebrafish were detected in the study population, based on an analysis of length-frequency data. The growth of *D. rerio* was modelled using the seasonalized Von Bertalanffy growth equation (Somers 1988), with parameters derived from length-frequency analysis. Growth parameters were: $L_\infty = 37$ mm, $K = 1.2$, $t_0 = -0.013$ y, $t_s = 0.4$ y, $C = 1$ (Fig. 1). The parameter C determines the intensity of seasonal oscillations in growth, having a value between 0 and 1. In addition, the growth rate (dSL/dt) of *D. rerio* with age was estimated using the differential form of the seasonalized Von Bertalanffy growth model (Fig. 1). Fish collected before October 2005 of SL > 15 mm were assumed to belong to the 2004 cohort, spawned towards the end of the season that year, and were excluded from the analysis.

Sex was not determined for all the fish used in the length-frequency analysis but in the subsample dissected for gut content analysis the sex ratio was 1:1. The mean (± SD) SL of males in the subsample was 20.4 ± 2.70 and females 21.5 ± 3.15; females were significantly larger than males (unpaired *t*- test, $\log_{10}$ transformed data: t_{297}, $P < 0.01$).

DISCUSSION

Diet

The zebrafish appears to feed chiefly on zooplankton in the water column, but may also feed from the substrate. The inclusion of food items from a terrestrial source suggests it may also consume food items that are on, or near, the water surface. These findings are consistent with previous experimental results which show that wild zebrafish occupy the entire water column (Spence *et al.*, 2006a, paper B). The findings of this study are largely in accordance with a diet study of zebrafish by Dutta (1993) and of eight danonine species by McClure *et al.* (2006), although both showed that insects formed a larger component of the diet than zooplankton. Dutta (1993) also included estimates of protozoa in the diet, which were not quantified in the present study. Further studies are required to determine the extent to which food items in the gut of zebrafish reflect selectivity on the part of the zebrafish as opposed to seasonal availability of different prey.

Growth and Recruitment

Based on length-frequency data, the zebrafish appeared to be primarily an annual species, with the main period of recruitment from April to August. There were two distinct age classes during the summer months, representing reproductively mature 1+ year fish and a cohort of 0+ year fish. The data suggest that growth and reproduction are linked to season, with the main period of rapid growth during the monsoon season, and the commencement of spawning just before the onset of the monsoon. However, larger females (*c.* 23 mm SL) collected in January were found to contain mature ova (Spence *et al.* 2006a, paper B), indicating that reproduction may not be cued solely by season, but may also be dependent on food availability, which is likely to be greatest during the monsoon months. This conclusion is consistent with the fact that domesticated zebrafish and wild-caught zebrafish in captivity breed all year round.

The maximum SL observed was 35 mm, which is comparable to the typical range observed in domesticated zebrafish of *c.*30-40 mm. Growth rates are considerably higher in domesticated populations; Eaton & Farley (1974a) reported an annual growth rate of 183 mm y^{-1} during the first 45 days of development, compared to 72 mm during the first two months in the study population (Fig. 1). In addition to being kept at constantly high temperatures (typically 25-29 °C) with regular feeding on high-quality food, domesticated populations may be subject to artificial selection, albeit inadvertent, for fast growth and early reproduction. Despite the difference in growth rates, wild and domesticated zebrafish appear to reach reproductive maturity at similar sizes. Length-frequency analysis showed that after one year the majority of fish in the study population measured 25 mm SL. In laboratory conditions, F_1 wild zebrafish also reach reproductive maturity at approximately 23 mm SL (R. Spence, pers. obs.), although they still exhibit slower growth rates than domesticated populations (Wright *et al.* 2006a, 2006b). Eaton & Farley (1974a) showed that in domesticated zebrafish maturity occurred after 75 days, when females were 24.9 mm SL and males 23.1 mm.

These results are consistent with the view that the zebrafish is an annual species. While it is possible that some fish may survive to breed a second year, there was no evidence of this in the study population. Among domesticated populations Gerhard *et al.* (2002) reported a mean life span of 42 months, with the oldest individual surviving for 66 months. However, instances of spinal curvature, a phenotype commonly associated with senescence (Gerhard *et al.* 2002), becomes apparent in domesticated and wild zebrafish after their second year in captivity (R. Spence, pers. obs.). Spinal curvature was not observed in the study population and it is likely that fish die in natural populations before this condition develops.

CHAPTER THREE: REPRODUCTIVE BEHAVIOUR

Paper D: Male territoriality mediates density and sex ratio effects on oviposition in the zebrafish

This paper is published in: *Animal Behaviour* 2005, **69** 1317-1323.

Authors: Rowena Spence and Carl Smith.

Abstract

Skew in the operational sex ratio of a population can have important consequences for mating competition and typically results in differential mating success. Population density can also influence mating behaviour and may interact with the operational sex ratio. This study investigated the effects of both density and operational sex ratio on male territoriality and female oviposition in the zebrafish. The sex bias was manipulated in favour of either males or females at two density levels and the impact on females of aggression and courtship behaviour by territorial males was measured in terms of egg production. Total egg production increased as a function of the number of females in each of the four treatments. However, mean *per capita* egg production was lower at high densities. It is proposed that this effect was due either to increased aggression at high densities by territorial males towards rivals, which interrupted female spawning attempts, or suppression of reproduction by dominant females. At high densities, territorial males maintained high rates of aggression regardless of sex bias, but courtship rates were significantly lower in male-biased treatments. It is further proposed that this response, whereby males constrain courtship in the presence of potential rivals, may be adaptive in the context of sperm competition avoidance.

INTRODUCTION

Sexual selection arises from competition over mates and mating opportunities, and frequently results in differential intrasexual mating success (Andersson 1994). The intensity of sexual selection is influenced by the local operational sex ratio (OSR): the ratio of males to females ready to mate at a given moment (Emlen & Oring 1977). The OSR is determined by three factors: the adult sex ratio in the population, the spatial and temporal distribution of the two sexes, and their potential reproductive rates (Clutton-Brock & Parker 1992). The rate at which a sex can potentially reproduce depends on the investment in gamete replenishment and parental care (Kvarnemo & Ahnesjö 2002). Even in species with no parental care, the OSR is usually skewed because females are limited by the number of eggs they can produce, whereas male reproductive fitness is limited by the number of eggs they can fertilise. Thus, males typically compete among each other for matings, whereas females are selective about mating partners.

Competition among males for matings takes various forms, including contests and alternative mating tactics (Warner & Hoffman 1980; Gross 1991), mate guarding (Enders 1993; Alonso-Pimentel & Papaj 1996) and sperm competition (Parker *et al.* 1996; Smith *et al.* 2002). Females may select mates on the basis of ownership of resources, in which case female choice acts in combination with male competition (Thornhill 1980), the possession of particular behavioural and morphological traits (Houde & Endler 1990) or an interaction between the two (Smith *et al.* 2002). The direction and degree of skew of the OSR in a population influences the amount of intrasexual competition and variance in male reproductive success. Enders (1993) showed that a male-biased OSR increased aggression among male spider mites, *Tetranychus urticae*; large males obtained greater reproductive success than small males. Grant *et al.* (1995) found that in Japanese medaka, *Oryzias latipes*, the frequency of male-male aggression, sneaking and disruption of courtship increased as the

OSR became more male-biased. Clutton-Brock *et al.* (1997) found that a female-biased OSR among red deer, *Cervus elaphus*, resulted in an increase in the proportion of males holding harems but a decrease in the number of days for which harems were held.

Population density also influences mating behaviour. For example, Jirotkul (1999a) found that male guppies decreased their courtship displays as density increased. However, changes in population density may entail concomitant changes to OSR, affecting the number of potential mates, the number of competitors, or both. Thus, population density comprises two factors: the absolute density of each sex and the density of males relative to females (Kvarnemo & Ahnesjö 2002). These factors are difficult to separate and may interact. Alonso-Pimentel & Papaj (1996) showed that copulation duration in the walnut fly, *Rhagoletis juglandis*, was affected by the density of each gender as well as the ratio of one to the other. Warner & Hoffman (1980) found that mating success among territorial male bluehead wrasse, *Thalassoma bifasciatum*, varied with population density, sex ratio and the numbers of non-territorial males, the effects being different on large and small reefs. The present study investigated the effects of varying density and sex ratio on female egg production and male reproductive behaviour in the zebrafish.

Under benign laboratory conditions zebrafish breed all year; spawning is influenced by photoperiod and takes place within approximately an hour of exposure to light following darkness (Darrow & Harris 2004). Although fecundity is high, reports differ as to the precise length of the female reproductive cycle and mean clutch size (Hisaoka & Firlit 1962; Eaton & Farley 1974b). Zebrafish are batch spawners; all mature ova are released during a single spawning session. Eggs are relatively large, 0.7 mm in diameter, and are released directly onto the substrate: hatching occurs between 48-72 h after fertilisation (Kimmel *et al.* 1995). Ovulation is dependent on female exposure to male gonadal pheromones (van den Hurk *et al.* 1987) while similar pheromones released by females after ovulation induce courtship behaviour in males (van den Hurk & Lambert 1983). Bloom & Perlmutter (1977) demonstrated that zebrafish produce pheromones that function as both inter and intrasexual attractants but it is not clear whether these have a role in reproduction or more generally in shoaling behaviour.

The aim of the study was to determine the effects of density and sex ratio on zebrafish spawning behaviour. Preliminary observations showed that female zebrafish may be choosy over spawning substrate, while males are territorial and aggressively defend access to spawning sites by other males. To investigate the consequences of male territoriality for female oviposition the study manipulated density and sex ratio and measured the impact of this behaviour in terms of rate of egg production.

It was predicted that *per capita* female egg production would decrease as a function of density, because of either increased aggression by the territorial male interfering with female oviposition or competition among females for access to suitable spawning areas. Differences in *per capita* egg production with sex bias might indicate whether one of these explanations was more probable. Both courtship and aggression by the territorial male were predicted to increase with density because of an increase in both available mates and potential rivals. Aggression was predicted to be higher with a male-biased sex ratio whereas courtship was predicted to be higher with a female-biased sex ratio.

METHODS

Experimental conditions

The experiment was conducted in February 2004, using 216 zebrafish obtained from a commercial supplier. The fish used were a stock that have been used for research on developmental genetics and showed no morphological abnormalities or unusual behaviour during pilot studies or during the experiment. Fish were housed in an environmentally controlled room with a 14:10 h light: dark cycle (0800 AM-1000 PM) which reflects day length during the breeding season in nature. The mean (± SD) SL of females in the study was 30 (± 1.9) mm and for males 30 (± 1.4) mm. The age of the fish was estimated on the basis of SL to be between 26 and 52 weeks. Females reach sexual maturity at about 11 weeks (Eaton & Farley 1974b). Experiments were conducted in 60 l glass aquaria (60 x 35 x 40 cm) with tap water (aerated for 24 h before the introduction of fish) at 26.5 - 28.5 °C. Water quality in aquaria was maintained using filters and aquaria water was oxygenated with an air stone. A single plastic box (150 x 100 x 40 mm), filled with 150 marbles, was placed in the front right-hand corner of every experimental aquarium as a spawning site. Zebrafish readily use boxes of marbles for oviposition; the eggs fall among the marbles, which prevents cannibalism. No other substrate was used. Throughout the experiment fish were fed three times each day with a mixture of frozen blood worm and high protein commercial salmon smolt pellets. Fish were not fed until after the daily spawning period and completion of behavioural observations.

Experimental design

The experimental design comprised two factors: density and sex ratio. The low-density treatment consisted of three fish and the high-density treatment 15 fish. Sex ratios (males/(males + females)) were biased in a 2:1 ratio towards either males (0.67) or females (0.34) at each density. Thus, there were four treatments: low density, male bias (two males, one female); low density, female bias (one male, two females); high density, male bias (10 males, five females); high density, female bias (five males, 10 females). The experiment was conducted over a three-week period, with a total of six replicates of each treatment. Each replicate lasted four days.

Prior to an experiment males and females were kept separately in 60 l aquaria. Males and females were randomly assigned to experimental treatments, with the sexes distinguished on the basis of body shape, females being more rounded ventrally. Any fish for which the sex could not be readily determined were excluded. Males were transferred to experimental aquaria three days before the experiment began to allow them to acclimatise to their new surroundings and to other males in their experimental group. Females were added to aquaria the evening before the experiment. Opaque dividers were placed between aquaria to prevent visual interactions between neighbouring fish. The exception was in the case of solitary males, where dividers were not installed until after the addition of females. Delaying putting the dividers in place facilitated solitary males becoming acclimatised to aquaria and served to prevent stress associated with their being housed alone.

Each aquarium was observed daily to determine whether there was a territorial male defending the spawning site. Individual territorial males were identified from unique distinguishing features of their colour pattern. Four replicates were videotaped for five min each morning for the four days of the experiment during the first 40 min after the lights came on. Thus, each replicate was videotaped on two of the four days for which behaviour was monitored. The frequency of aggression and courtship behaviours by the territorial male were scored from video footage. Definitions of these behaviours are set out in Table I. Each instance of either of the two aggressive behaviours were scored as one instance of aggression. No distinction was made between aggression directed towards males or females, because this could not be reliably scored in high-density treatments. In the case of courtship, each instance of initiation of a courtship attempt was scored as one instance of courtship, although not all the components listed in Table I would necessarily be observed on each occasion. Where the territorial male left the spawning site and pursued the female around the entire tank, each instance of returning to the spawning site was scored as a new courtship attempt.

Spawning sites were removed after 1030 AM each morning and all the eggs deposited in them were carefully removed, counted and then incubated as part of a separate genetic study. Spawning sites were replaced 3-4 h after removal.

On day five of the experiment, all experimental fish were removed from the aquaria and killed with an overdose of clove oil. Fish were measured to the nearest 0.1 mm and dissected to confirm sex.

Table I. Aggressive and courtship behaviours recorded for territorial male zebrafish

Behaviour	**Definition**
Aggression	
Repel	Male swims rapidly towards another fish (male or female), which changes speed or direction.
Circle	Male and a second male approach one another with dorsal and anal fins erect, they orientate head to tail and slowly circle each other while ascending.
Courtship	
Chase	Male swims towards a female, often touching her with his snout. The male may continue to follow the female for an extended period.
Lead	Male approaches a female and swims in a tight circle or figure of eight around or in front of her. If the female does not follow the male, he may alternate between circling the female and swimming back and forth between the female and the spawning site.
Parallel	Once over the spawning site, the male swims alongside the female, in contact but slightly behind her, his head approximately level with her operculum.
Quiver	Male oscillates his body at high frequency and low amplitude close to the female over the spawning site.

Data analysis

All data were tested for normality using a Kolmogorov-Smirnov test and for equality of variance using a Bartlett's test. Data on egg production and aggression were square-root transformed before analysis. Courtship and aggressive behaviour by the territorial male are presented as rates *per* 1 min. The mean scores for observations for each replicate were used in analysis to avoid pseudoreplication. Two-factor ANOVAs were used to test the effects of density and sex bias on egg production and male courtship and aggressive behaviour. Where there were significant interaction effects, unpaired *t*-tests were used to make further comparisons between individual factors. To test for changes in the rate of aggression within treatments during the experiment a repeated measures ANOVA was conducted, with treatment as the independent variable and day as the repeated measure. Because data did not respond to standard transformations, they were ranked and the rank transformation statistic calculated as a non-parametric equivalent (Kepner & Robinson 1988; Zar 1996). A paired *t*-test was used to compare the mean difference in SL between territorial males and other males within replicates.

RESULTS

Of the 216 fish used in the experiment, sex was determined incorrectly in five individuals over three replicates, all of which were in the high-density female-biased treatment. Although this changed the sex ratio from 0.34 to 0.4 and 0.47 respectively, it did not alter the direction of the sex bias.

Female zebrafish proved capable of spawning every day, with a mean clutch size of 52.3 (± 8.84) eggs in the low-density male-biased treatment, which included only a single female. Total egg production increased as a function of both density (two-way ANOVA: $F_{1,20} = 28.06$, $P < 0.001$) and sex ratio (two-way ANOVA: $F_{1,20} = 10.12$, $P = 0.005$). The mean total number of eggs produced by females was greatest in the high-density treatment and with a female-biased sex ratio (Fig. 1a). There was no interaction between these factors (two-way ANOVA: $F_{1,20} = 0.48$, $P = 0.497$). Female *per capita* egg production was significantly higher at low densities (two-way ANOVA: $F_{1,20} = 5.82$, $P = 0.026$; Fig. 1b), but there was no effect of sex bias (two-way ANOVA: $F_{1,20} = 0.17$, $P = 0.688$) and no interaction between density and sex ratio (two-way ANOVA: $F_{1,20} = 1.50$, $P = 0.235$).

In all treatments with more than one male present, a single male established a territory around the artificial spawning site and remained in possession of the territory throughout the four days of the experiment. The mean (± SD) SL of territorial males (30.8 (± 1.34) mm) was significantly greater than that of other males in the same replicate (29.8 (± 1.08) mm) (paired *t*-test: $t_{17} = 3.59$, $P < 0.01$).

Territorial male aggression rate differed significantly between density (two-way ANOVA: $F_{1,20} = 85.11$, $P < 0.001$; Fig. 2a) and sex ratio treatments (two-way ANOVA: $F_{1,20} = 35.56$, $P < 0.001$), and there was also a significant interaction between these factors (two-way ANOVA: $F_{1,20} = 7.94$, $P = 0.011$). At high fish density there was no significant effect of sex bias on aggression rate (unpaired *t*-test: $t_{10} = 1.68$, $P = 0.120$). During the experiment there was a significant difference in rates of aggression between treatments (two-way ANOVA: $F_{2,24} = 6.73$, $P = 0.005$) but within treatments aggression did not differ significantly over time (two-way ANOVA: $F_{3,24} = 2.06$, $P = 0.132$). There was a significant interaction effect (two-way ANOVA: $F_{6,24} = 2.63$, $P = 0.042$), owing to aggression increasing over time in the high-density female-biased treatment (Fig. 3). The low-density female-biased treatment was excluded from this analysis because no aggression was recorded.

The courtship rate of territorial males was significantly higher in female-biased treatments (two-way ANOVA: $F_{1,20} = 19.52$, $P < 0.001$; Fig. 2b) but was not significantly different between density treatments (two-way ANOVA: $F_{1,20} = 1.13$, $P = 0.300$). There was a

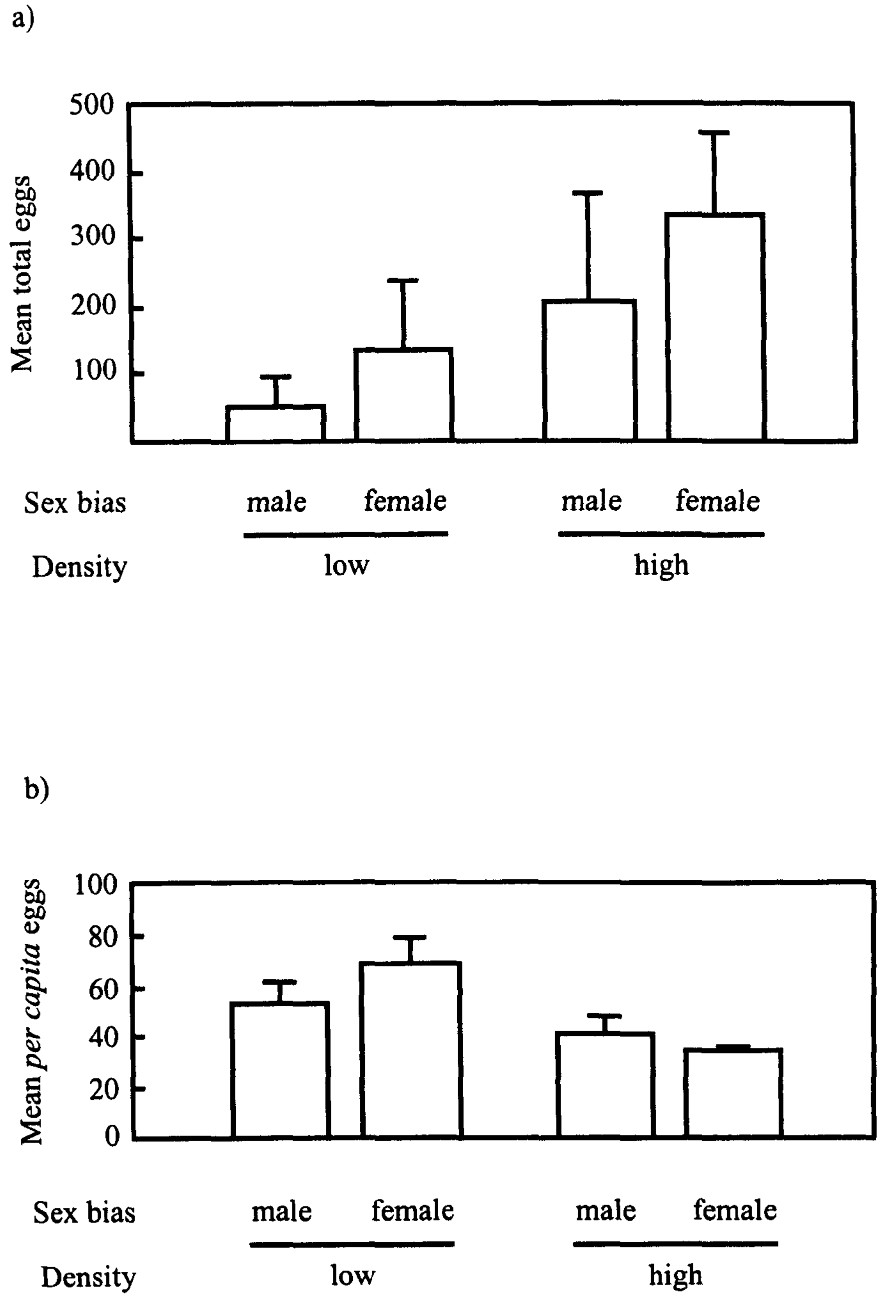

Figure 1. Mean +SE (a) total and (b) *per capita* egg production of zebrafish under four treatments: low density, male bias (two males, one female); low density, female bias (one male, two females); high density, male bias (10 males, five females); high density, female bias (five males, 10 females).

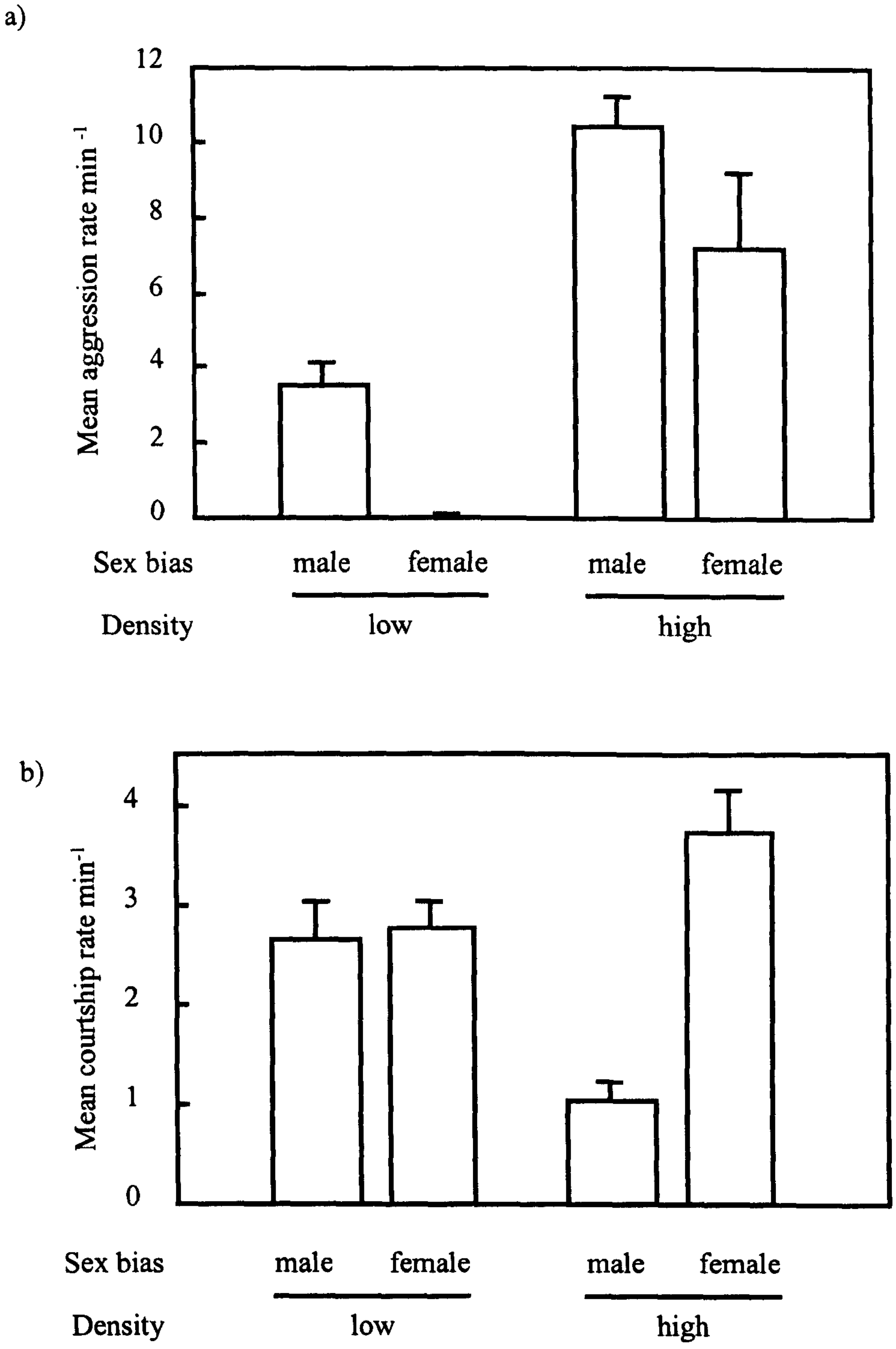

Figure 2. Mean +SE rate of (a) aggression and (b) courtship by territorial male zebrafish under four treatments: low density, male bias (two males, one female); low density, female bias (one male, two females); high density, male bias (10 males, five females); high density, female bias (five males, 10 females).

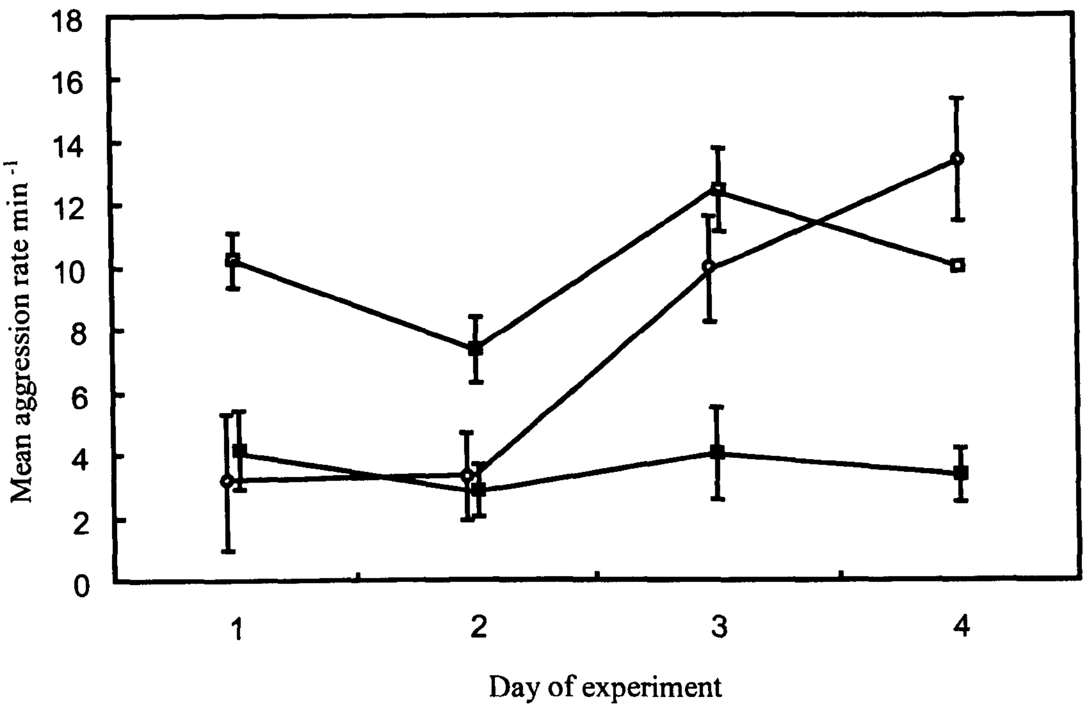

Figure 3. Mean +SE rate of aggression over the course of the experiment under three treatments: □ : high density, male bias; ○ : high density, female bias; ■ : low density, male bias.

significant interaction between sex ratio and density on courtship rate (two-way ANOVA: $F_{1,20}$ = 16.46, P = 0.001); in the male-biased treatments courtship was significantly lower in the high density treatment (unpaired t-test: t_{10} = 3.76, P = 0.004) and in the high-density treatments, courtship rate was significantly lower in the male-biased treatment (unpaired t-test: t_{10} = 5.95, P <0.001). At high female densities, territorial males maintained high levels of both aggression and courtship (Fig. 2).

There was no overall correlation between territorial male courtship and aggressive behaviour (Pearson correlation, r_p = -0.289, N = 24, P = 0.170), though there was a significant negative correlation between courtship and aggression in male-biased treatments (Pearson correlation: r_p = -0.741, N = 12, P = 0.006).

DISCUSSION

In all replicates, regardless of density or sex ratio treatments, a single male established and maintained a territory, throughout the experiment, around the artificial spawning site provided. This is the first time male territoriality has been reported in zebrafish reproductive behaviour. This study contrasts with that of Pyron (2003) who proposed that male zebrafish do not display territoriality or courtship behaviour, although he did note that males showed aggression towards each other.

Total egg production increased as a function of the number of females present in each treatment (Fig. 1a). However, mean *per capita* egg production decreased at higher densities (Fig. 1b). This effect may have arisen because the aggressive behaviour of the territorial male towards rival males may have interrupted female oviposition and constrained spawning frequency. There is also evidence for reproductive suppression of subordinates by dominant females (Gerlach 2006). Another possibility is that competition among females constrained access to suitable spawning sites. The fact that a male defended the spawning site implies that it was perceived as a superior site for oviposition. Some females may have deposited their eggs outside the spawning sites provided (R. Spence, pers. obs.) in which case the eggs would be quickly eaten by other fish. Thus, the significant density effect recorded on mean *per capita* egg production may be a direct effect of density on oviposition, or indirectly on egg survival. Both clearly have consequences for female oviposition and reproductive success.

Interference with spawning attempts at high densities was not directly observed but is implied by the increased rate of aggression (Fig. 2a). In other fish species, experimental manipulation of fish density has been shown to lead to increased male-male competition, with a resultant reduction in the opportunities for females to spawn. For example Grant *et al.* (1995) observed male Japanese medaka inspecting, bumping and trying to separate spawning pairs. Reichard *et al.* (2004a) found that female European bitterling, *Rhodeus amarus*, abandoned spawning attempts when males interrupted courtship to attack rivals at high densities, with the result that total spawning rate in the study population was significantly reduced.

An alternative explanation for the lower rate of aggression recorded at low densities is that familiar individuals are less aggressive (Griffiths *et al.* 2004). However, all fish were equally familiar at the start of the experiment, and there was no reduction in aggression rates during the experiment (Fig. 3). Thus, the difference in rates of aggression recorded between the high and low-density treatments seems to result from an increase in the number of rivals at high densities rather than as a consequence of familiarity at low densities.

Rates of courtship by the territorial male were significantly lower in the high-density male-biased treatment than in any other (Fig. 2b). At low densities territorial males actively courted females, periodically returning to the spawning site. In contrast, at high densities territorial males confined their activities to within a few body lengths of the spawning site, vigorously defending the area from other males (R. Spence, pers. obs.). This observation may be an indication that at high densities territorial defence takes priority over mate acquisition, as suggested by Warner & Hoffman (1980). Thus the effect of sex bias on courtship rates at high densities can be partially explained in terms of differential encounter rates.

However, in the male-biased treatments, courtship rate decreased with increasing density, even though there were more females present. Although this could be interpreted as a trade-off between courtship and aggression, in the female-biased treatment at high density, males maintained high rates of both (Fig. 2), indicating that the two are not mutually exclusive activities. At high densities aggression was equally high in both male and female-biased treatments whereas courtship was reduced only in the male-biased treatment. This effect may reflect a response to the risk of sneaked matings by other males, with territorial males constraining their courtship behaviour in the presence of potential rivals which may attempt to fertilise eggs without incurring the energetic costs of courtship.

In other fishes males have been shown to adjust their behaviour in response to the risk of sneaking and sperm competition. For example, Jirotkul (1999b) found that male guppies decreased their courtship displays at male-biased OSRs. Le Comber *et al.* (2003) showed that male three-spined sticklebacks, *Gasterosteus aculeatus*, increased their rate of zigzag courtship displays when presented with a solitary female but not when presented with a female in the presence of a rival male. Smith *et al.* (2003) showed that European bitterling avoided leading females to spawning sites in the close proximity of a rival male, and concluded that the close proximity of rivals to the spawning site may serve as a proximate cue for assessing sperm competition intensity.

The extent to which these findings reflect the natural spawning behaviour of zebrafish is not clear, although a field-based mesocosm study with wild-caught zebrafish showed that their mating behaviour is comparable (Spence *et al.*, paper G). As far as group size is concerned, zebrafish form small shoals of 2-20 fish in the wild (Spence *et al.* 2006a, paper B). Observations conducted in a large aquarium showed that females spent more time in association with one or two males than in a larger shoal (Delaney *et al.* 2002) and the findings of the field-based mesocosm study (Spence *et al.*, paper G) suggest that this is also the case in nature. In a study of the spotted goby, *Gobiusculus flavenscens*, Forsgren *et al.* (2004a) found that variation in sex ratios during the breeding season led to marked variation in mating competition. It is likely that similar variation would apply among zebrafish populations between the wet and dry seasons.

In conclusion, two clear results emerge from this study. First, males are highly territorial and the reproductive success of males and females may be affected by territoriality. Second, both sex ratio and density affect male territorial behaviour and thereby female reproductive success.

Paper E: Genetic analysis of male reproductive success in relation to density in the zebrafish

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Authors: Rowena Spence, William C. Jordan & Carl Smith

Abstract

In this study, behavioural and genetic data were used to investigate the effects of density on male reproductive success in the zebrafish. Based on previous measurements of aggression and courtship behaviour by territorial males, it was predicted that they would sire more offspring than non-territorial males. Microsatellite analysis of paternity showed that at low densities territorial males had higher reproductive success than non-territorial males. However, at high density territorial males were no more successful than non-territorials and the sex difference in the opportunity for sexual selection, based on the parameter I_{mates}, was low. Male zebrafish exhibit two distinct mating tactics; territoriality and active pursuit of females. Male reproductive success is density dependent and the opportunity for sexual selection appears to be weak in this species.

INTRODUCTION

The advent of genetic parentage analysis has had a substantial impact on our understanding of animal mating systems. Many socially monogamous species have proven to be genetically polygamous (Birkhead 1987), while territorial or harem-holding males have frequently been shown to be cuckolded (DeWoody & Avise 2001). Moreover, due to the operation of sperm competition (Birkhead & Møller 1998) and cryptic female choice (Eberhard 1996), mating success is not equivalent to reproductive success. It is now recognised that genetic analyses are crucial for measuring parentage, and thereby in understanding mating system evolution and the strength of sexual selection (Avise 1994; Hughes 1998; Reichard *et al.* 2005).

The strength of sexual selection depends on the sex difference in the degree of variance in reproductive success for each sex; the greater the difference between the sexes, the more opportunity there is for selection to operate (Shuster & Wade 2003). In most species the variation in male mating success, as defined by mate number and offspring number, exceeds that of female mating success, as defined by clutch size and number of clutches. While females tend to produce similar numbers of offspring, variance in the number of offspring fathered among males can be high, with successful males monopolising females and some males failing to reproduce. The sex difference in the opportunity for sexual selection can be quantified using the parameter I_{mates} (Shuster & Wade 2003); the difference in male and female variance in reproductive success as a function of the squared mean reproductive success for each sex:

$$I_{\mathrm{mates}} = I_{\mathrm{males}} - I_{\mathrm{females}}$$

where

$$I_{\mathrm{males}} = \frac{V_{\mathrm{males}}}{X^2_{\mathrm{males}}}$$

and

$$I_{\mathrm{females}} = \frac{V_{\mathrm{females}}}{X^2_{\mathrm{females}}}$$

if V = variance in reproductive success, and X^2= squared mean reproductive success.

The greater the value of I_{mates}, the greater the opportunity for sexual selection, which is typically reflected in the degree of sexual dimorphism. However, variance in male success can be eroded through alternative male mating tactics, sperm competition and cryptic female mate choice (Lifjeld *et al.* 1994; Jones *et al.* 2001).

The intensity of competition for mates is influenced both by the number of competitors and the temporal and spatial distribution of fertilisations (Emlen & Oring 1977; Shuster & Wade 2003). When sexually receptive females are spatially clustered, it is easier for a single dominant male to monopolise matings to the exclusion of other males. In addition, if fertilisations are distributed evenly through time, rather than clustered, a small number of males may be able to monopolise matings. Variance in male reproductive success also correlates negatively with male density. As male density increases, rates of cuckoldry increase and the fitness payoff of territoriality relative to alternative mating tactics is lower (Philipp & Gross 1994; DeWoody & Avise 2001; Reichard *et al.* 2004b). This study used behavioural and molecular analysis to investigate the effects of density on male mating success in the zebrafish.

Male and female zebrafish are similar in size and colouration. Under laboratory conditions zebrafish breed all year. Female zebrafish will release eggs directly onto a bare substrate, but when provided with an artificial spawning site, such as a plastic box filled with gravel or marbles, will preferentially use it for oviposition. Some male zebrafish are territorial during mating and a single male will aggressively attempt to control access of rivals to a spawning site (Spence & Smith 2005, paper D). Both territorial and non-territorial males show the same courtship behaviour, but whereas non-territorial males pursue females all around the aquarium, territorial males confine their activities to within a few body lengths of the spawning site and chase other males away when they try to approach.

In the previous study density and sex ratio were manipulated and aggressive and courtship behaviour by territorial males was measured under each treatment (Spence & Smith 2005, paper D). Rates of aggression increased as a function of density, although courtship did not (Spence & Smith 2005, paper D). The aim of the present study was to investigate the effects of density on territorial male reproductive success, using the offspring produced in the behavioural experiment (Spence & Smith 2005, paper D). It was predicted that territorial males would have lower reproductive success at higher densities, due to increased competition from rival males. In addition, in the high-density treatment, the sex difference in the opportunity for sexual selection was measured. For zebrafish, which show little sexual dimorphism, a low estimate of I_{mates} was predicted.

METHODS

Experiment

The experimental design and results of behavioural analyses are presented in Spence & Smith (2005, paper D), though no results are repeated here. The original experimental design comprised two factors: density and sex ratio. However, only density effects were investigated in the present study. The low-density treatment consisted of three fish, one female and two males (six replicates) and the high-density treatment of fifteen fish, either ten males and five females or five males and ten females (six replicates of each).

Parentage analysis

All the adults and a subset of 31-32 offspring from each replicate were genotyped. The mean (± SD) daily number of eggs per replicate was 52 ± 8.8 in the low density treatment and 270 ± 28.5 in the high density treatments. A total of 198 adults and 560 offspring were genotyped. Offspring from a single day's spawning were selected haphazardly from each replicate. DNA was extracted from fin samples or embryos with the yolk sac removed, using Promega Wizard SV 96 Genomic DNA purification system.

Individuals were screened across seven microsatellite loci (Z10914, Z1213, Z1233, Z1496, Z6454, Z669 and Z851) belonging to six different linkage groups, with a mean of 7 alleles per locus. Primer sequences were taken from the zebrafish genetic map website (http://zebrafish.mgh.harvard.edu/papers/zf_map/primers.html; Knapik *et al.* 1996, 1998). DNA was amplified in a 6 μl reaction volume containing 1 μl genomic DNA; 2 pmol dye-labelled forward primer; 2 pmol reverse primer and 4 μl Qiagen Multiplex PCR Master Mix. PCR cycling consisted of an initial 12 min denaturation at 95 °C, 30 cycles of 30 s at 94 °C, 2 min at 58 °C and 1 min at 72 °C and a final extension at 60 °C for 30 s. 1 μl PCR product was mixed with 10 μl dionized formamide and 15 μl LIZ 500 size standard and run on an automated sequencer, Applied Biosystems 3100 Genetic Analyzer. Fragment length was determined using Genemapper 3.5 software. All adults were genotyped 3 times in order to control for the effects of null alleles and allelic dropout (Bonin *et al.* 2004). The error rate (the ratio of observed allelic differences to total allelic comparisons among repeated amplifications) was estimated to be 3.4%.

Parentage was assigned using CERVUS 2.0 software (Marshall *et al.* 1998). Observed heterozygosities ranged from 0.20 to 0.71. In the low-density treatment, where maternal genotype was known, paternity was assigned with 95% confidence in 92% of the offspring sampled, (range 78%-100% between replicates). In the high-density treatments where maternity was not known, CERVUS was used in a stepwise manner, determining parentage first for the sex with the smallest number of candidate parents in a given replicate (i.e. females in the male-biased treatment and males in the female-biased treatment) and using those data where parentage was assigned with 95% confidence to determine parentage for the other sex (Marshall *et al.* 1998). Paternity was assigned with 95% confidence to 244 of 380 offspring genotyped and with 80% confidence to a further 107, (range 60%-100% between replicates). Maternity was assigned with 95% confidence to 205 of the offspring and with 80% confidence to a further, 137, (range 78%-100% between replicates). Thus the combined exclusionary power for the high-density treatments was 83%.

Data analysis

All data were tested for normality using a Kolmogorov-Smirnov test and for equality of variance using Bartlett's test. The reproductive success of males was calculated as a percentage of offspring sired within a replicate, paternity being assigned for each individual male, both territorial and non-territorial. Only data where paternity could be assigned with at least 80% confidence were used in the analysis. A two factor ANOVA was used to test for the effects of territoriality and sex bias on male reproductive success in the high-density treatments. Paired *t*-tests were used to test for a difference in reproductive success between territorial and non-territorial males (mean value per male) within each density level. Following the recommendation of Nakagawa (2004), Bonferroni corrections were not applied and instead a measure of effect size (Cohen's *d*) was estimated. This index measures the magnitude of a treatment effect as the standardised difference between two means by comparing the overlap in the distribution between the two data sets independently of sample size. An effect size of 0.8 is defined as large (Cohen 1988).

RESULTS

In the low-density treatment territorial males sired a mean ± SD of 56.3% ± 7.58 of the offspring, a significantly greater proportion than non-territorial males at 43.8% ± 7.58 (paired *t*-test: $t_5 = 3.05$, $P = 0.028$, $d = 1.76$) (Fig.1). In the high-density treatments there was no effect of either territoriality (two-way ANOVA: $F_{1,20} = 6.39$, $P = 0.304$) or sex bias (two-way

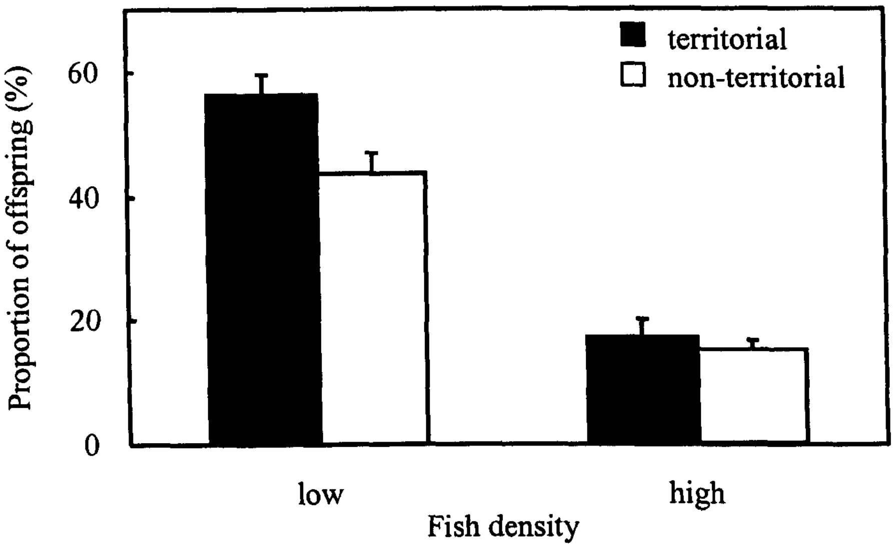


Figure 1: The mean proportion (%) + SE of offspring sired by territorial and non-territorial male zebrafish under two density treatments.

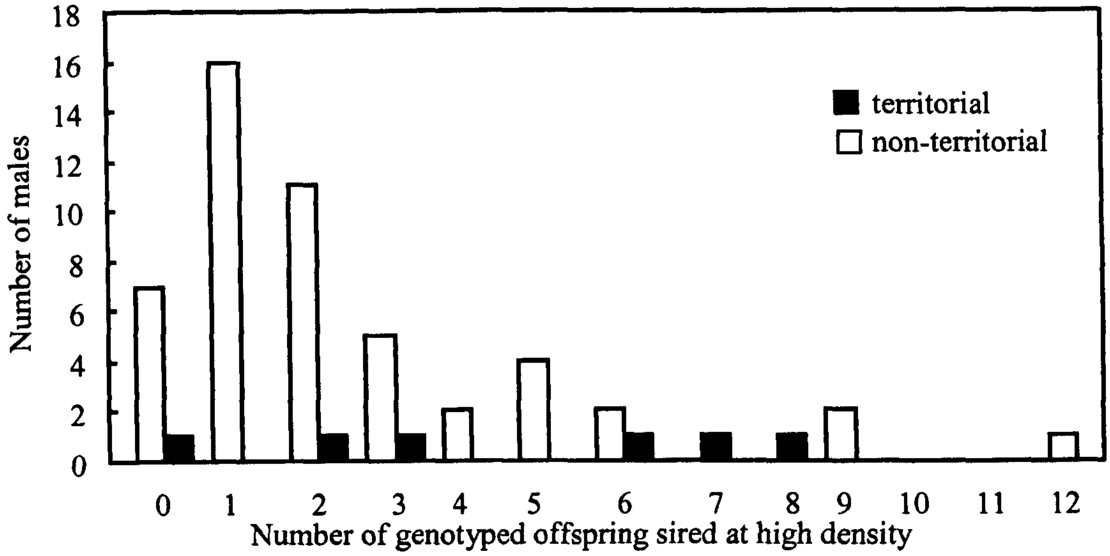


Figure 2: The frequency distribution of genotyped offspring among territorial and non-territorial males in the high-density male-biased treatment.

ANOVA: $F_{1,20} = 12.3$, $P = 0.159$) on the number of offspring sired per male, nor was there an interaction (two-way ANOVA: $F_{1,20} = 5.21$, $P = 0.352$). In the high-density male-biased treatment territorial males sired a mean ± SD of 17.1% ± 12.91 of the offspring, compared to 83.0% ± 12.91 by non-territorial males, a mean of 9.2% ± 9.79 offspring per non-territorial male. In the high-density female-biased treatment the territorial male sired a mean ± SD of 16.9% ± 11.02 of the offspring, compared to 83.3% ± 10.72 by non-territorial males, a mean of 20.8% ± 14.82 per non-territorial male. Both high-density treatments were then combined, thereby doubling the sample size; there was still no significant difference in reproductive success between territorial and non-territorial males; territorial males sired a mean ± SD of 17% ± 11.4 of the offspring, compared to 83% ± 11.4 by non-territorial males, a mean of 15% ± 6.4 per non-territorial male (paired *t*-test: $t_{10} = 0.48$, $P = 0.639$, $d = 0.223$) (Fig. 1).

In the high-density male-biased treatment the majority of experimental fish of each sex were represented in the parentage analysis. In total, 87% of males sired at least one of the 30 genotyped offspring (range 0-12, Fig. 2). Females produced a mean ± SD of 14.8% ± 3.57 of the offspring each and 89% of the females in each replicate produced at least one of the 30 genotyped offspring. The estimated opportunity for sexual selection was low; $I_{\text{males}} = 0.83 \pm 0.22$ and $I_{\text{females}} = 0.80 \pm 0.25$, giving an estimate of 0.03 for I_{mates}. In the low density treatment where there was only one female, $I_{\text{females}} = 0$, so $I_{\text{males}} = I_{\text{mates}} = 0.07 \pm 0.01$.

DISCUSSION

Despite the wealth of genetic studies conducted on zebrafish, this is the first time a genetic parentage analysis has been applied to understanding their mating system. This study also serves as an example of how genetic analyses can provide insights that are not obvious from behavioural studies alone. The study showed that at a low density territorial males sired significantly more offspring than non-territorial males (Fig.1). While this result is consistent with the previous observation that at low densities territorial males monopolise spawning sites (Spence & Smith 2005, paper D), non-territorial males were still able to achieve a relatively high reproductive success. The overall rate of paternity assignment was high (92%) and while it is possible that the paternity of the remaining unassigned 8% could erode the significance of this result, it is unlikely that the pattern of paternity would differ significantly from that observed in the majority that were assigned, given that non-assignment of offspring is likely to be random across potential sires.

At higher densities no significant difference was detected in the reproductive success of territorial and non-territorial males in either sex ratio treatment. The combined data from both high-density treatments were then analysed, thereby doubling the number of replicates, which increased the statistical power of the test and also yielded a non-significant result. While aggressive territoriality had been predicted to confer some fitness advantage at higher densities, it had also been predicted that the advantage would be negatively correlated with density. As the fitness advantage at low densities is not great it is unsurprising that it should be eroded at higher densities. However, this result does raise the question of how territorial behaviour is maintained through selection. Both density levels used were within the range of densities at which zebrafish occur in nature and territorial defence also occurs under natural conditions (Spence *et al.*, paper G). Given that territoriality only occurs during the brief daily spawning period, the possible fitness cost associated with energy expenditure on territoriality may not exceed that for courtship. Consequently, while territorial defence confers a fitness advantage at low densities, it may not always do so at high densities, though the reproductive fitness of territorial males may be at least equal to that of non-territorial males. In addition, the adoption of one or other tactic, territorial defence or active pursuit of females, may be frequency dependent, in which case they would be predicted to confer equal fitness payoffs.

Alternative mating tactics are common in fish, partly because of the prevalence of external fertilisation, which makes it hard to exclude rivals (Taborsky 2001). In genetic studies of several nest-guarding species with paternal care Dewoody & Avise (2001) found that between 5-30% of embryos in nests were not sired by the nest owner. In some species, such as Atlantic salmon, *Salmo salar,* and bluegill sunfish, *Lepomis macrochirus*, territorial and sneaker males are morphologically distinct (Gross 1982). In other species, such as the three-spined stickleback (Rico *et al.* 1992) and the European bitterling, (Reichard *et al.* 2004a; Smith *et al.* 2004a) the adoption of one or other role is flexible and probably frequency dependent. Although each territorial male in this study maintained his territorial role throughout the 4 days of the experiment, territorial male zebrafish are not morphologically distinct. Further, on the basis of laboratory and field observations, the frequency of territorial behaviour is influenced by the availability of high quality spawning sites, a factor that was not manipulated in the current study (Spence *et al.*, paper G).

It should be noted that fertilisation rates in zebrafish may not always reach 100%, though this was not recorded in the current study. Thus, the results reflect the reproductive success of males as determined by differential embryo survival to hatching (three days post-fertilisation) rather than fertilisation success. The genetic analysis also showed that the majority of females in each replicate produced offspring. The previous study (paper D) showed that females produce fewer eggs *per capita* at high densities, but in the absence of paternity analysis it was not clear whether this was because each female produced fewer eggs, or whether some females were excluded from spawning. This analysis suggests that females spawn smaller clutches at higher densities.

The estimate of I_{mates} indicates that there is a low opportunity for sexual selection in zebrafish; there was no significant difference in the variance in reproductive success between males and females. Because this estimate was based on genetic parentage data, it takes account of both behavioural differences (such as territoriality) and post-mating forms of sexual selection (sperm competition and cryptic female choice), which can impact on variance in reproductive success. This result is consistent with the lack of a marked sexual dimorphism in this species. Variance in male mating success is dependent on the temporal and spatial clustering of females; the opportunity for selection is predicted to be highest when receptive females show low temporal but high spatial clustering (Shuster & Wade 2003). Although zebrafish spawn almost daily under laboratory conditions, spawning is confined to an approximately one-hour period each day; i.e. matings are highly temporally clustered. In nature, spawning is more seasonal, but is similarly largely confined to a brief period at dawn (R Spence, pers. obs.). Spatial clustering of females may occur where sites for oviposition are limiting. However, although both females and males discriminate among spawning locations (Spence & Smith, paper G) they use a broad range of oviposition sites. Consequently, under natural conditions males may not always be able to monopolise either receptive females or sites of reproduction, with the outcome that the opportunity for sexual selection is weak.

The results of this study suggest that zebrafish have a promiscuous mating system with a low opportunity for sexual selection. Parentage analysis indicates that territoriality confers a fitness benefit at low densities, but at higher densities has an equivalent fitness payoff to non-territorial behaviour.

Paper F: Mating preference of female zebrafish in relation to male dominance

This paper is published in: *Behavioral Ecology* 2006, **17** 779-783.

Authors: Rowena Spence and Carl Smith.

Abstract

Mating success tends to be skewed toward dominant males, though female mate preferences may not always correlate with male dominance. This study investigated the mating preferences of female zebrafish in the absence of male-male competition. Females were paired sequentially with males of known dominance rank, using a nested, repeated-measures design, with egg production as a measure of female mate preference. It was predicted that females would spawn more frequently and produce larger clutches when paired with males of higher dominance rank. Significant differences were found among females in the size of clutches produced and among males in the size of clutches received, but these differences were independent of male dominance rank. Male body size was not related to either dominance rank or clutch size received. These results indicate that females vary clutch size in relation to the males with which they are paired but that they do not favour dominant males. Thus, male competition may normally override female mate preference in zebrafish.

INTRODUCTION

Sexual selection, that is, selection arising through competition over mates and matings, results from differential mating success among males (Darwin 1871). The strength of sexual selection depends on the sex difference in the variance in mating success for each sex; the greater the difference, the more opportunity there is for selection to operate (Shuster and Wade, 2003). Variance in male reproductive success arises through competition among males for mates and female mating preferences (Andersson 1994).

Despite the considerable research effort directed toward both of these mechanisms, their relative contribution to sexual selection is poorly understood (Qvarnström & Forsgren 1998; Wong & Candolin 2005). Traditionally, the two processes were viewed as complementary, females preferring male traits that are correlated with dominance, such as red in three-spined sticklebacks or body size in Japanese medaka (Howard *et al.* 1998; Candolin 1999). However, in other cases male competition may reduce opportunities for female choice. In the speckled cockroach, *Nauphoeta cinerea,* the composition of social pheromones preferred by females differs from that which signals male dominance (Moore & Moore 1999), whereas in European bitterling, male courtship vigour is a better predictor of female preference than dominance (Reichard *et al.* 2005). Thus, although mating success tends to be skewed in favour of dominant males, the fitness outcomes to females can vary across taxa (Qvarnström & Forsgren 1998; Moore *et al.* 2001; Wong & Candolin 2005).

Female preference can be difficult to demonstrate experimentally in species in which male competition plays a significant role in the mating system; where animals are allowed to interact freely, females may mate with the dominant male by default. To control for the effects of male competition, dichotomous choice designs are often employed, with preferences inferred from the amount of time a female spends in association with males differing in a particular trait. There are a number of problems with such designs. First, presenting females simultaneously with two extremes of the trait in question can result in amplification of a preference effect (Wagner 1998). Under natural conditions, variation in a trait is more likely to be normally distributed. Further, female preference is multifactorial and any trait selected *a priori* may be correlated with other, uncontrolled traits. Second, many studies do not take account of within- and between-female variations in preferences. Third, preference is inferred rather than demonstrated empirically. Although association time has been shown to correlate with mating preference in some species, in others it does not (Fuller 2003). Finally, females may employ cryptic forms of choice, such as variation in the rate of oviposition, differential allocation to eggs and post-copulatory sperm selection (Burley 1986, Lifjeld *et al.* 1994, Eberhard 1996). The present study used egg production to evaluate female mate preference in the zebrafish.

Zebrafish typify a basic mating pattern common to many cyprinid fishes; they are group spawners and eggs scatterers (Breder & Rosen 1966). However, females appear to be choosy with respect to sites for oviposition (Spence *et al.* paper G). Moreover, some males are territorial during the daily spawning period and attempt to monopolize access to oviposition sites, which may affect female reproductive success (Spence & Smith 2005). The aim of the present study was to establish whether female zebrafish exhibit mating preferences independently of male-male interactions, and whether these correlate with male dominance. It also aimed to address the problems associated with dichotomous choice designs outlined above. First, males were ranked according to dominance, using four ranks rather than just two. Then females were paired sequentially with males of different dominance ranks, allowing them the opportunity to spawn in the absence of competition. Each pairing was repeated three times, and clutch size was used as a measure of female mating preference. Preliminary observations showed that there is wide variation in clutch size both within and among female zebrafish, suggesting that females might modulate oviposition rate in relation to their spawning partners. Clutch size adjustment is one of the few modes of cryptic choice available to species with external fertilization (Eberhard 1996) and has been demonstrated across a range of taxa (Côte & Hunt 1989; Rintamaeki *et al.* 1998; Arnqvist & Danielsson 1999; Reyer *et al.* 1999; Parker 2003).

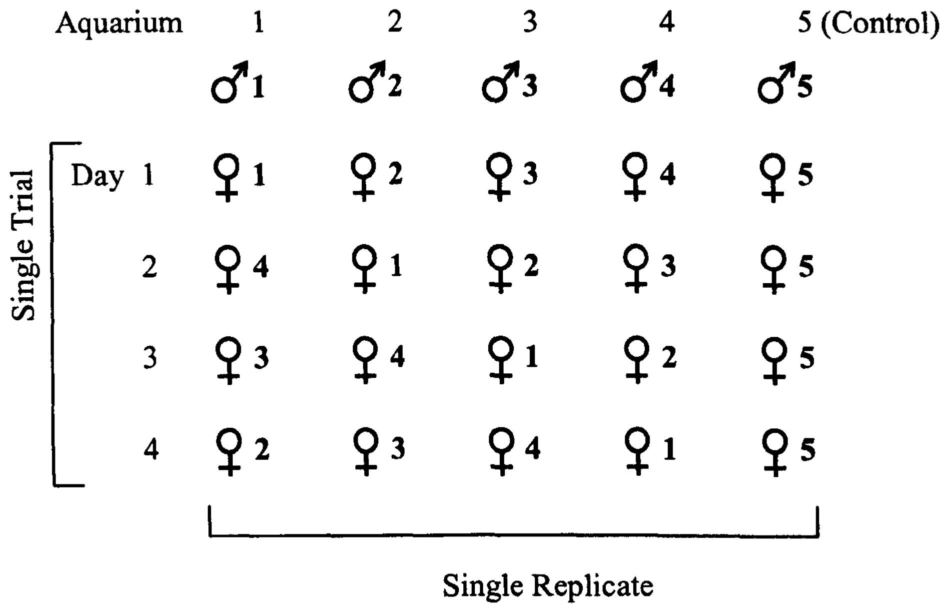
The study tested the prediction that females spawn more frequently and produce larger clutches when paired with males of higher dominance rank. Because females were paired with different males, clutch size could vary independently between males and females. As female preference may be independent of male dominance, rejection of the null hypothesis would be dependent on demonstrating a significant difference among males in clutch size received, and that such a difference was related to male dominance rank. The repeated-measures design increased the chances of any such effect being detected.

METHODS

The experiment was conducted in August 2004, using 30 male and 30 female zebrafish, obtained from commercial suppliers. Fish were housed in an environmentally controlled room with a 14:10 h light: dark cycle (0800 AM-1000 PM) with water on a recirculating system at 27 °C. Throughout the experiment fish were fed three times each day with a mixture of frozen bloodworm, brine shrimp and high protein commercial fish pellets (Cyprico Crumble Excellent). The mean ± SD SL of males was 35 ± 1.8 mm and females 36 ± 2.3 mm. Prior to the start of the experiment males and females were kept separately in 60 l aquaria (60 x 40 x 40 cm). Males and females were randomly assigned to treatment or control groups, females being distinguished by the presence of a genital papilla.

The experimental design consisted of pairing females with males of different dominance ranks and counting the number of eggs they produced. Male dominance rank was determined as follows: each group of four males was placed in a 60 l glass aquarium together with four haphazardly selected females. A single plastic box (150 x 100 x 40 mm), covered with 2 mm nylon mesh and four plastic plants, was placed in the back left hand corner of each aquarium as a spawning site. Male zebrafish readily establish and defend territories around such artificial spawning sites; a territorial male typically emerges at the start of the first spawning period and remains in possession of the territory over successive days (Spence & Smith, 2005, paper D). On the morning of the following day the fish were observed during the first hour after the lights came on and the territorial male in each group was identified and removed. This procedure was repeated on subsequent days to identify the second, third and fourth ranked males. None of the females were reused in the next stage of the experiment.

After ranking, males were randomly assigned to adjacent 20 l glass aquaria (40 x 30 x 25 cm), where they remained throughout the experiment. Opaque barriers were placed between aquaria to prevent visual interactions between neighbouring fish. Each aquarium was lined with aluminium mesh (mesh size 6 x 3 mm) to protect eggs from cannibalism after spawning. Four plastic plants were placed on top of the mesh. On the day after completion of the dominance ranking exercise, females were randomly assigned to males. A nested, repeated-measures design was used with six replicates, each comprising four females and four ranked males; each female was paired with each of the four males on three separate occasions (Fig. 1). Each female was placed with one male for 24 h, and then assigned to the next male. This process was repeated for 12 days. A Latin square design was used to randomize the order in which females were paired with males while ensuring that each female was placed with a different male each day for four days. In addition a fifth pair of fish was included in each replicate and left together for the entire duration of the experiment in order to partially control for any effects on the spawning cycle due to daily reassignment of treatment females.



Aquarium	1	2	3	4	5 (Control)
	♂1	♂2	♂3	♂4	♂5
Day 1	♀1	♀2	♀3	♀4	♀5
2	♀4	♀1	♀2	♀3	♀5
3	♀3	♀4	♀1	♀2	♀5
4	♀2	♀3	♀4	♀1	♀5

Figure 1. Diagrammatic representation of the experimental design: males remained in the same aquaria and females (other than controls) were paired with each male in turn. There were six replicates and three trials in total.

Each morning the fish, mesh and plants were removed from each aquarium and it was swept with a fine mesh hand net to collect all the eggs that had been spawned during the past 24 h. Males were then returned to the same aquarium and females assigned to the next male. This procedure was carried out between 0915 AM and 1200 noon each day. The eggs were counted and fixed in 4% formalin. The same protocol was followed for control pairs, though in this case the same fish were always replaced together.

At the end of the experiment the fish were measured to the nearest 0.1 mm. One of the males died on the second day of the experiment, so this replicate was restarted with a replacement male and a further dominance ranking exercise that included the replacement male.

Data analysis

All data were tested for normality using a Kolmogorov-Smirnov test and for equality of variance using Bartlett's test. To test for male and female influence on variance in clutch size, the Scheirer-Ray-Hare extension of the Kruskal-Wallis test was used, as a non-parametric equivalent of the two-way ANOVA (Sokal & Rohlf, 1995). This procedure involves ranking the data, performing a two-way ANOVA, and testing the ratio H (computed as SS/MS_{total}) as a χ^2 variable. A nested, repeated-measures design was used, with experimental fish nested within replicates and trial as a repeated measure. Female inter-spawning interval and body size correlated significantly with clutch size ($P < 0.05$) and were used as covariates in the analysis. A Mann-Whitney U-test was used to compare egg production between treatment and control females and an unpaired t-test to compare body size. A Chi-squared test was used to test whether the number of clutches obtained by males differed in relation to male dominance rank, with the null expectation that the number of clutches did not differ among dominance ranks. A correlation was used to test for a relationship between male dominance rank and clutch size. A nested one-way ANOVA was used to test for a difference in male body size among dominance ranks, together with a correlation between dominance rank and ranked body size within replicate.

RESULTS

The mean ± SD clutch size (i.e. eggs spawned in each 24-h period) was 185 ± 149.5 for treatment females and 169 ± 146.0 for controls. The mean ± SD daily number of eggs spawned over the entire 12 days (i.e. including days without spawning) was 116 ± 148.4 for treatment females and 103 ± 140.4 for controls. The maximum clutch size observed was 765. There was no significant difference between treatment and control females in either clutch size (Mann-Whitney U-test: $U = 3408$, $N_1 = 42$, $N_2 = 180$, $P = 0.322$), mean daily number of eggs (Mann-Whitney U-test: $U = 9402$, $N_1 = 69$, $N_2 = 288$, $P = 0.476$), or female body size (unpaired t-test: $t_9 = 1.770$, $P = 0.110$).

All experimental females spawned, though some failed to spawn with some males. On average, treatment females spawned at least once with 3 out of their 4 mates while none of the controls failed to spawn. The mean ± SD inter-spawning interval of treatment females was 1.6 ± 0.85 days (range 1-6) and control females 1.5 ± 0.60 days (range 1-3). One of the treatment females spawned on each of the 12 days while the maximum number of spawnings among control females was 8. Clutch size did not decrease during the course of the experiment (Pearson correlation $r_p = -0.496$, $P = 0.101$). Clutch size was correlated with inter-spawning interval for both treatment (Spearman's rank correlation: $r_s = 0.287$, $N = 180$, $P < 0.001$) and control females (Pearson's correlation: $r_p = 0.387$, $N = 42$, $P = 0.011$).

The number of spawnings obtained by treatment males ranged from 3-10, compared with a range of 5-8 spawnings for control males. There was no significant difference in body size among male dominance ranks (one-way nested ANOVA: $F_{3,20} = 0.436$, $P = 0.730$), neither was there a correlation between dominance rank and ranked body size within replicate (Spearman's rank correlation: $r_s = 0.00$, $P = 1.00$). There was no correlation between male body size and mean clutch size received (Pearson's correlation: $r_p = -0.281$, $N = 24$, $P = 0.183$).

After controlling for inter-spawning interval and female body size as covariates, there was a significant difference among treatment females in the size of clutches produced, (Scheirer-Ray-Hare test: $\chi^2_{54} = 1158$, $P < 0.001$). There was also a significant difference among treatment males in clutch size received (Scheirer-Ray-Hare: $\chi^2_{54} = 1194$, $P = < 0.001$). In light of the reservations of Toothaker & Chang (1980) concerning the Scheirer-Ray-Hare test, and following their recommendation, a parametric analysis was conducted on untransformed data, testing male and female factors separately and using the covariates only when testing for a female effect on clutch size. In this case no significant female effect was detected on clutch size (ANCOVA: $F_{54,\ 214} = 1.07$, $P = 0.356$), though there was a significant male effect

(ANOVA: $F_{54, 216} = 1.41$, $P = 0.045$). The distribution of clutches did not differ significantly among dominance ranks ($\chi^2_3 = 0.580$, $P = 0.900$), and there was no correlation between ranked clutch size and dominance rank within replicate (Spearman's rank correlation $r_s = 0.270$, N = 24, $P = 0.208$). Thus, although females did spawn more frequently and produce larger clutches with some males than others, this effect was not related to dominance rank.

DISCUSSION

The aim of this study was to investigate female mating preferences in the absence of male-male competition in zebrafish, with a prediction that females would prefer dominant males. After controlling for the effects of inter-spawning interval and female body size, there was a significant difference in the size of clutches produced among treatment females. There was also a significant difference among treatment males in clutch size received. These results suggest that females consistently spawn more frequently and produce larger clutches with some males than others. However, this effect was not related to male dominance rank. The design employed in this study allowed male and female contributions to variance in clutch size to be separated. It should be noted that the significant male effect appeared to be stronger than the female effect, since the female effect was not detected in a parametric analysis of untransformed (but non-normal) data. As noted above, it is the male effect which is of interest in this study, since egg production might differ among females for reasons other than the male they are paired with, in addition to differing as a function of the variables which were identified and controlled for as covariates.

The ability of females to vary egg production in response to different mating opportunities has been demonstrated in a number of fish species. In the redlip blenny, *Ophioblennius atlanticus,* both the probability of spawning and the number of eggs released was greater with larger than smaller males (Côte & Hunt 1989). In Chinook salmon, *Oncorhynchus* *tshawytscha,* where males provide no parental care, females were observed to delay spawning successive clutches when paired with smaller males (Berejikian *et al.* 2000). Also, Fu *et al.* (2001) found that female bluegill sunfish produce more eggs in the presence of sneaker males, as do female European bitterling, (Smith & Reichard 2005). In a previous study of zebrafish spawning behaviour, individual females paired with two males simultaneously spawned daily for four days, a higher rate than that observed in the present study among either treatment or control females (Spence & Smith 2005, paper D). Variation in egg volume has also been proposed as a form of female mate preference in fishes (Kolm 2001), but was not measured in the present study.

The experimental design employed three trials in order to control for the natural spawning cycle of females, although the results show that females are capable of spawning every day for at least 12 days. Females sustain comparable spawning rates in nature (Spence *et al.*, paper G), although spawning is more seasonal (Spence *et al.* in press, paper C). Twenty-four hours was sufficient duration for each trial; Eaton & Farley (1974b) showed that exposure of female zebrafish to a male, even for a few hours, was sufficient to induce ovulation. Moreover, ovulation only takes place during mating (Hisaoka & Firlitt 1962), so there was little risk of preferences being affected by previous suitors (Bakker & Milinski 1991). The repeated-measures design of the experiment, with randomization of the order of pairing, also controlled for any such effects.

The study controlled for any effects of disturbance due to the daily changes of mate by using a control group of fish that were paired for the duration of the experiment, but which experienced daily capture and egg removal. Although there was no difference in mean egg production between treatment and control fish, the treatment fish showed a greater range in the number of times they spawned. This variation may indicate increased variance in egg production in the treatment group in response to changes in mates, though it could also be a function of a larger sample size of the treatment group.

The failure to detect a correlation between male rank and number and size of clutches received indicates that male competition may normally override female mate preference in zebrafish. If dominance does play a role in determining mating preferences among female zebrafish, this does not appear to relate to cues which females can detect in isolated males. Studies with both three-spined sticklebacks (Candolin 1999) and sex role-reversed pipefish, *Sygnathus typhle,* (Berglund & Rosenqvist 2001) have shown that signal expression can change during competition and thus affect choice. However, in both these species female preference correlates with male dominance. A number of studies in other species have shown that females do not necessarily prefer dominant males; male dominance does not always predict mate quality and there may be fitness costs associated with choosing a dominant male (Qvarnström & Forsgren 1998, Holland & Rice 1999; Byrne & Roberts 2004).

No relationship was found between male body size and either clutch size received or male dominance rank, although in a previous study territorial male zebrafish were found to be larger than non-territorials (Spence & Smith 2005, paper D). The failure to detect a relationship between male size and dominance rank may be because the design only allowed for an effect to be tested within replicates, effectively reducing the sample size to six. However, the lack of a relationship between male body size and clutch size received is a robust result, supported by a large sample size and repeated measures design. This finding appears to contradict that of Pyron (2003), who showed that females prefer to associate with larger males. The discrepancy between studies probably reflects differences in experimental design; Pyron (2003) used a dichotomous choice test with two groups of males selected on the basis of size differences, whereas this study used males selected at random from an experimental population without large size variation, and focused on differences in dominance. The possibility that females might show a preference for larger males when compared simultaneously with smaller males cannot be excluded, or that dominance and size correlate in males.

While the present study suggests that female zebrafish exhibit mating preferences that are independent of male dominance, it is unclear how these preferences can be maintained. One possibility is that under natural conditions not all matings involve male competition. Another is that dominant males may not, in fact, gain significantly superior reproductive fitness (Spence *et al.* 2006b, paper E). The fact that spawning is confined to a brief period at dawn results in high temporal clustering of receptive females, allowing little opportunity for males to monopolize females (Emlen & Oring 1977). Although alternative mating tactics have traditionally been viewed as undermining female choice (Taborsky 1998; Jones *et al.* 2001), this will not be the case if sneaker males are those preferred by females, as has been shown in European bitterling (Smith & Reichard 2005).

Further research is necessary to determine the mechanism underlying the female preferences observed in this study. Assortative mating may relate to genetic compatibility. Incompatibility avoidance has been proposed as influencing female mating preferences and may promote polyandry as an adaptive mating strategy (Zeh & Zeh 2003). Assortative mating based on genetic compatibility can reduce the intensity of sexual selection on males. Investigating interactions between maternal and paternal genomes in the zebrafish may be a fruitful avenue for further research as it is a tractable laboratory species and its genetics are well understood (Grunwald & Eisen 2002). In view of the role played by pheromones in the reproductive behaviour of both sexes it is likely that mating preferences may be based on olfactory cues (Gerlach 2006).

In conclusion, both male-male competition and female preference may operate in the zebrafish mating system, although female preference was not correlated with male dominance in the present study. Instead, the two mechanisms may operate in opposition, resulting in a low overall opportunity for sexual selection. Further research is needed to examine the significance of female oviposition decisions in the zebrafish mating system and the role of olfactory cues in determining the basis of mate preference.

Paper G: Adaptive oviposition decisions are mediated by spawning site quality in the zebrafish

This paper has been submitted to *Ethology*.

Authors: Rowena Spence, Rebecca Ashton and Carl Smith

Abstract

In oviparous species a female's choice of oviposition site can have a significant impact on offspring survival and thereby on reproductive fitness. This study compared mating behaviour of domesticated zebrafish in the laboratory with wild fish in a field-based study in Bangladesh. Spawning site characteristics were manipulated in order to examine oviposition decisions by females. In a laboratory experiment zebrafish were provided with a choice of four discrete spawning sites containing gravel or silt, with or without vegetation. The behaviour of females and territorial males was observed during spawning and the number of eggs deposited in each spawning substrate was recorded. A gravel substrate was preferred by both males and females and vegetation was preferred over non-vegetated substrates. The experiment was repeated in Bangladesh and yielded the same substrate preference, although no effect of the presence of vegetation was detected. When eggs were incubated under all four treatments, it was shown that egg survival was greatest in gravel, suggesting an adaptive explanation for zebrafish oviposition decisions.

INTRODUCTION

In oviparous species, choice of oviposition site can have a significant impact on offspring survival and thereby on reproductive fitness (Huey 1991; Wootton 1998) and may influence life history evolution (Resetarits 1996). Potential oviposition sites may vary in quality, or may already contain eggs of other individuals, including other species. Females must also choose whether to deposit all their eggs together or across several sites. The fitness consequences of oviposition site decisions may be as important to females as choice of mate and there are a number of studies focussing on the relative contributions of oviposition site quality and male characteristics. Species in which oviposition site quality has been found to play a central role in female mate choice include pied flycatchers, *Ficedula hypoleuca* (Alatalo *et al.* 1986), spotted wrasse, *Notolabrus celidotus,* (Jones 1981) and bluehead wrasse (Warner 1987). In other species, there may be an interaction between the direct benefits of oviposition site quality and the indirect benefits of male quality (Smith *et al.* 2002). Even in species with male parental care, female oviposition decisions may be influenced by a variety of both biotic and abiotic factors in addition to parental investment. For instance, in some fish species, females prefer to spawn with males whose nests already contain eggs. Various adaptive explanations have been put forward for this including mate choice copying (Goulet 1998), nest concealment reducing predation risk (Kraak *et al.* 2000), reduced risk of filial cannibalism (the dilution effect) (Forsgren *et al.* 2004b), as well as environmental parameters, such as oxygen conditions within the nest (Reynolds & Jones 1999; Payne *et al.* 2002).

In contrast, zebrafish are group spawners and egg scatterers with no parental care. Although females do express mate preferences (Spence & Smith 2006, paper F), the opportunity for sexual selection appears to be weak in this species and they do not display striking sexual dimorphism (Spence et. al. 2006b, Paper E). Choice of spawning site is one of the few ways in which females of such species can maximise offspring survival. Zebrafish eggs are demersal and non-adhesive. Some male zebrafish are territorial during mating and males will aggressively attempt to control access by rival males to a spawning site (Spence & Smith 2005, paper D), thereby increasing their probability of fertilising eggs (Spence *et al.* 2006b, paper E). Thus, if females actively choose oviposition sites, males may increase their reproductive success by guarding such sites. The present study manipulated spawning site characteristics in order to examine oviposition decisions by female zebrafish in the laboratory and field.

Spawning behaviour has never been described in wild zebrafish, nor observed in the field. Thus it is not known whether features such as light cuing, spawning over artificial substrates and male territoriality are natural behaviours or laboratory artefacts. A laboratory choice experiment was conducted with domesticated fish and replicated in a field-based mesocosm experiment with wild fish in Bangladesh. The study tested for male and female oviposition preferences by offering a choice between gravel and silt as spawning substrates. Silt is the predominant substrate in the natural habitat of zebrafish in Bangladesh (Spence *et al.* 2006a, paper B). However, eggs deposited on silt may be easily buried, where they risk suffocation. A gravel substrate, in contrast, allows water to circulate through the interstice so that eggs are oxygenated. In addition, eggs deposited among gravel are protected from cannibalism and predation. Salmonids bury their eggs in loose gravel (Eliott 1994) and many egg-scattering cyprinids spawn preferentially on gravel substrates (Breder & Rosen 1966).

It was predicted that males would preferentially guard gravel-substrate spawning sites and that females would deposit more eggs in gravel than on silt. It was also predicted that eggs incubated in gravel would have higher survival than those incubated in silt. In addition, the study tested for an effect of vegetation on spawning preferences. Vegetation is another common feature of the zebrafish natural habitat (Spence *et al.* 2006a, paper B). Zebrafish larvae possess attachment apparatus and are able to adhere to plants following hatching until inflation of the swim bladder (Laale 1977). Vegetation may also serve to protect larvae from predation. Thus, it was further predicted that males and females would prefer vegetated to non-vegetated spawning sites.

METHODS

Laboratory choice experiment

The experiment was conducted during May 2005, using 136 zebrafish (mean ± SD SL 32 ± 2.3 mm) bred in the laboratory from stock obtained from a commercial supplier. Fish were housed in an environmentally controlled room with a 14:10 h light: dark cycle (0800 AM-1000 PM) and fed three times each day with a mixture of frozen *Artemia* and high protein commercial fish pellets (Cyprico Crumble Excellent). Experiments were conducted in glass aquaria (60 x 40 x 40 cm) on a recirculating system with a water temperature of 25 °C. Four plastic boxes (150 x 100 x 40 mm), each comprising a different spawning substrate treatment, were placed according to a randomly determined protocol, one in each corner of each experimental aquarium. Substrates consisted of either 300 g of gravel (mean particle size approx. 1.5 cm) or 10 mm depth of autoclaved pond silt. In addition, 10 aquatic plants, *Vallisneria spiralis,* were placed in one box each of gravel and silt. *V. spiralis* is structurally similar to plants typically found in the natural habitat of zebrafish (Spence *et al.* 2006a, paper B) and is readily available from aquarist suppliers. Thus, there were four spawning site treatments: gravel, silt, gravel with vegetation and silt with vegetation.

On the day preceding the start of the experiment four male and four female zebrafish were released in each experimental aquarium in the late morning after the end of the spawning period for that day. On the following day the behaviour of fish in a subset of aquaria was observed during the spawning period. The presence of females and territorial males at each spawning site was recorded every 10 s for 10 min. The position of females was only recorded when they swam down onto the substrate either to spawn or inspect the spawning site.

The experiment was terminated immediately after spawning behaviour ceased, in order to prevent egg cannibalism. The fish and spawning sites were removed from the aquaria and the number of eggs in each spawning site recorded. In order to ensure all eggs were recovered from the silt, a lid was placed over the spawning site prior to its removal from the aquarium. The eggs were then pipetted off the surface of the silt and finally the silt was sieved through a fine mesh net. After removal of the spawning sites, each aquarium was swept with a fine mesh hand net to collect any eggs that had been deposited outside the spawning sites. At the end of the experiment the fish were measured to the nearest 0.1 mm and retained in the aquarium but were not used in the experiment again.

Egg survival

A further experiment was conducted to determine the effects of the different spawning site treatments on egg survival. Spawning sites were set up in 16 aquaria as described above but without adding fish. Eggs recovered from the previous experiment were gently pipetted onto the substrate. Eggs were randomly allocated to treatments, 50 eggs being incubated in each site. The set-up was completed between 2-4 h post-fertilisation. After three days the spawning sites were covered and removed from the aquaria, and the number of embryos surviving to hatching was counted.

Field-based mesocosm choice experiment

A larger scale choice experiment was conducted at the field station of the Faculty of Fisheries of the Bangladesh Agricultural University, Mymensingh, Bangladesh in August 2005, at the height of the natural zebrafish spawning season. The field station includes a number of natural ponds in which zebrafish are abundant. However, during the monsoon season the water was too turbid to enable direct behavioural observations. Therefore experiments were conducted in specially constructed large, glass-sided outdoor aquaria (246 x 30 x 84 cm) filled with pond water to a depth of 60 cm. Environmental conditions (light, temperature, rainfall, water quality) were identical to those in the surrounding ponds from which wild experimental fish were collected. Water temperature ranged from 29-33 °C. During mid-afternoon, rice-straw screens (108 x 208 cm) were placed on top of the aquaria to provide shade. Four spawning sites, identical to those used in the laboratory experiment, were placed at 50 cm intervals along the centre of the aquarium, in a randomly determined order. Gravel was the same as that used in the laboratory experiment, while silt and plants were collected from adjacent ponds.

Each aquarium was stocked with 30 fish captured from adjacent ponds with a fine mesh seine net. This density matched densities in the adjacent ponds (Spence *et al.* 2006a, paper B). The experiment used 420 fish in total, with a mean ± SD SL of 24 ± 3.6 mm. A 1:1 sex ratio was used in experimental aquaria. Sexing of fish was based on visual inspection. In a previous study, visual inspection was confirmed on dissection to be 98% accurate (Spence & Smith 2005, paper D). In addition, in a separate field study a subsample of fish taken from the same population was also confirmed on dissection to have a 1:1 sex ratio (Spence *et al.* in press, paper C). Fish were fed twice daily with a mixture of zooplankton captured from adjacent ponds and commercial dry food. The experiment was conducted using the same protocol as in the laboratory experiment, with a total of 14 replicates. Each aquarium was observed for 10 min during the spawning period which commenced at dawn, approximately 0530 AM. The experiment was conducted over six days with a total of 14 replicates. At the end of the experiment, the fish were measured to the nearest 1 mm and released back into the ponds from which they were collected.

Data analysis

All data were tested for normality using a Kolmogorov-Smirnov test and for equality of variance using Bartlett's test. Proportional data were arcsine transformed before analysis (Zar

1996). Chi-squared 2 x 2 contingency tests with Yate's correction were used to test for an association between substrate type, presence of vegetation and both the number of eggs spawned, and the spatial distribution of males and females. A two-way ANOVA was used to test for the effects of spawning site treatment on egg survival. Paired *t*-tests were used to compare the density of eggs deposited in spawning sites compared to that on the floor of aquaria.

RESULTS

In the laboratory choice experiment there was a significant association between the number of eggs spawned and substrate type and presence of vegetation (Chi-squared: $\chi^2_1 = 91.98$, $P < 0.001$). The observed numbers of eggs deposited in gravel, and vegetated sites irrespective of substrate, exceeded expected values (Fig. 1). The mean (± SD) density of eggs in spawning boxes was 49.1 ± 24.68 10 cm^{-2}. This was significantly higher than the density elsewhere in aquaria, 0.1 ± 0.07 10 cm^{-2} (paired *t*-test, $t_{15} = 7.93$, $P < 0.001$).

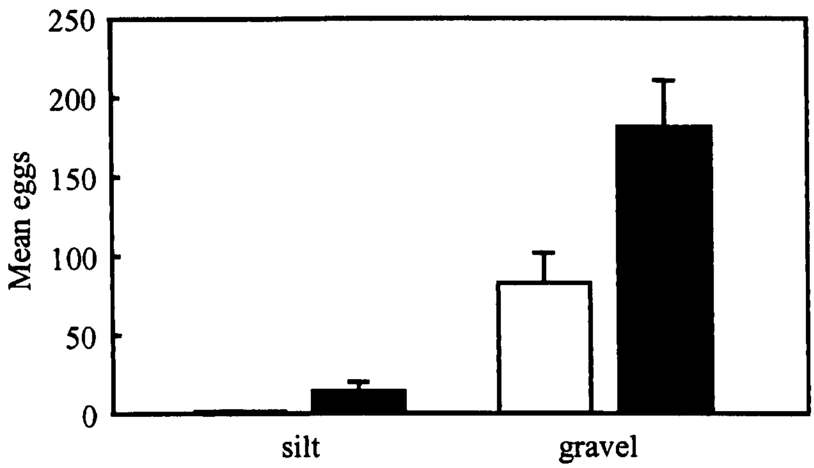


Figure 1:Laboratory choice experiment: Mean + SE eggs recovered from artificial spawning sites in each of four habitat types: gravel or silt substrate, ■ with vegetation or ☐ without.

In the laboratory choice experiment there was a significant association between habitat type and the spatial distribution of both territorial males (Chi-squared: $\chi^2_1 = 22$, $P < 0.001$, Fig. 2a) and females (Chi-squared: $\chi^2_1 = 215$, $P < 0.001$, Fig. 2b). Territorial males spent more time defending spawning boxes with a gravel substrate than silt, but did not preferentially defend vegetated sites. Females spent more time over gravel than silt substrate spawning sites; they also spent more time over vegetated than non-vegetated silt though there was no difference in the amount of time spent over gravel with or without vegetation.

There was a significant effect of substrate type on the proportion of eggs that survived to hatching (two-way ANOVA: $F_{1,63} = 19.23$, $P < 0.001$; Fig. 3). There was no effect of vegetation on survival (two-way ANOVA: $F_{1,63} = 0.14$, $P < 0.711$) and no interaction between substrate and presence of vegetation (two-way ANOVA: $F_{1,63} = 0.24$, $P < 0.629$).

In the field-based choice experiment, no eggs were deposited on silt. However, the number of eggs deposited in the vegetated and non-vegetated gravel sites did not deviate from expected (Chi-squared: $\chi^2_1 = 0.53$, $P = 0.468$, Fig. 4). The mean ± SD density of eggs inside spawning boxes was 5.1 ± 7.99 10 cm^{-2}, significantly higher than on the floor of experimental aquaria at 0.1 ± 0.12 10 cm^{-2} (Wilcoxon: $T = 105$. $N = 14$, $P = 0.001$).

In the field-based choice experiment there was a significant association between habitat type and the spatial distribution of territorial males (Chi-squared: $\chi^2_1 = 37$, $P < 0.001$, Fig. 5a) who spent more time defending gravel than silt substrate spawning boxes, though they did not preferentially defend vegetated sites. There was no overall association between substrate type and the spatial distribution of females (Chi-squared: $\chi^2_1 = 1$, $P = 0.262$, Fig. 5b).

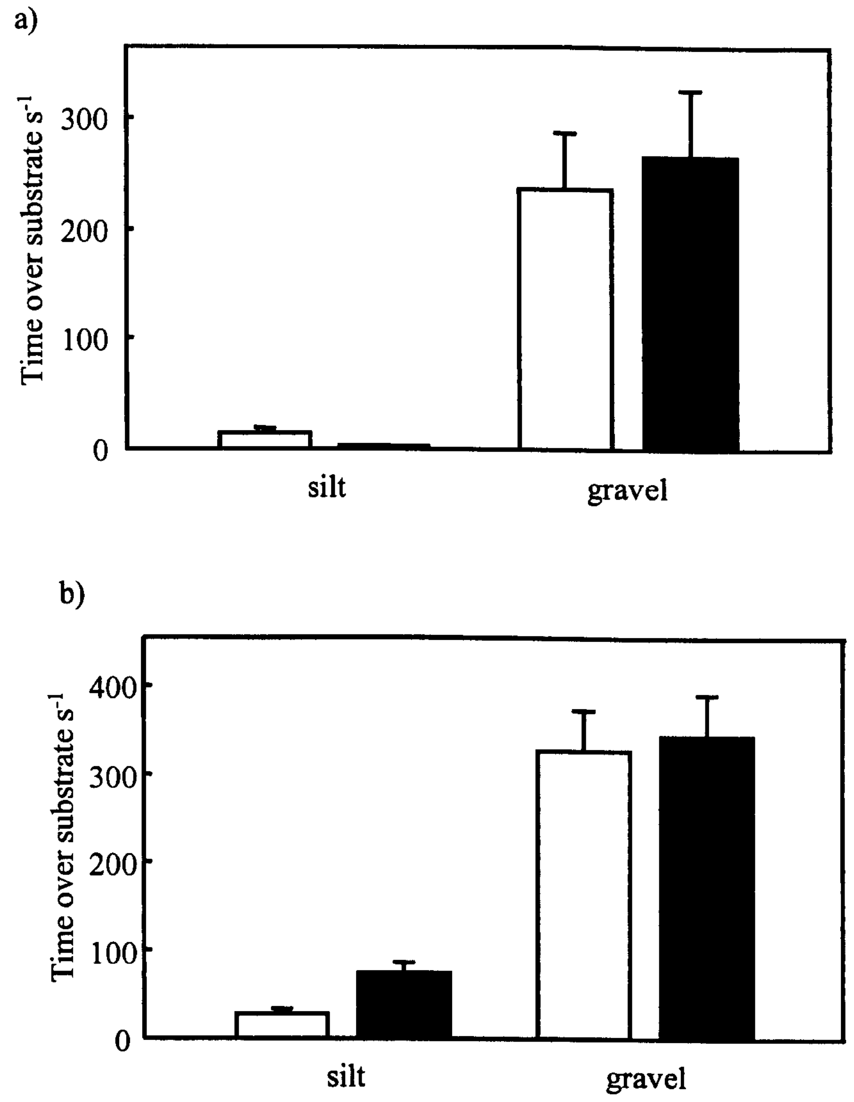


Figure 2: Laboratory choice experiment: Mean time (seconds) spent by a) males and b) females over artificial spawning sites in each of four habitat types: gravel or silt substrate, ■ with vegetation or □ without, in a 10 minute observation period.

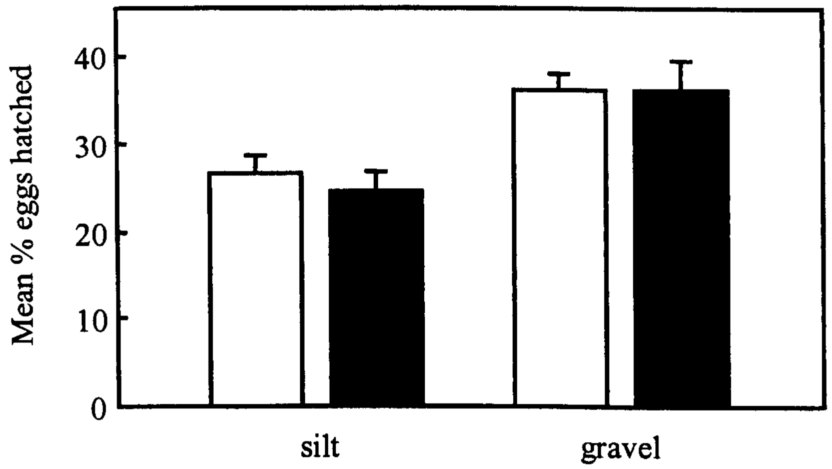


Figure 3: Egg survival: Mean + SE proportion (%) of eggs surviving to hatching under each of four habitat types: gravel or silt substrate, ■ with vegetation or ☐ without.

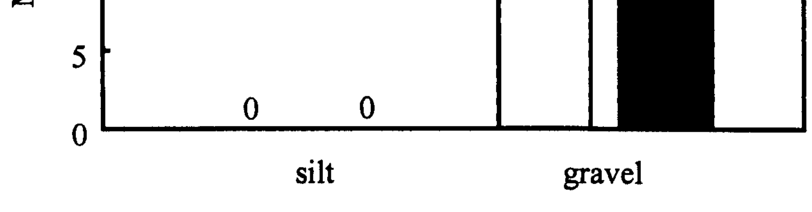


Figure 4: Field-based choice experiment: Mean + SE eggs recovered from artificial spawning sites in each of four habitat types: gravel or silt substrate, ■ with vegetation or ☐ without.

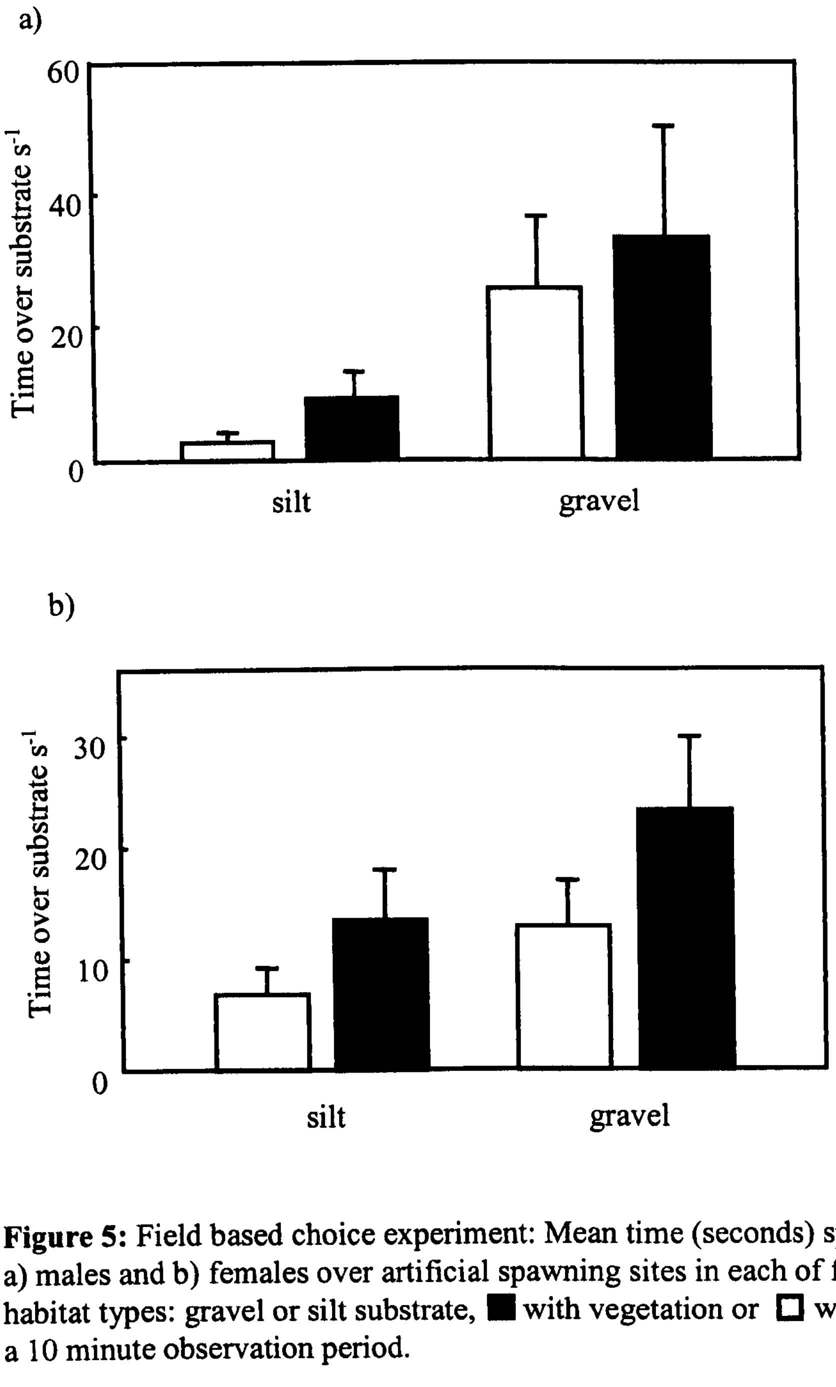


Figure 5: Field based choice experiment: Mean time (seconds) spent by a) males and b) females over artificial spawning sites in each of four habitat types: gravel or silt substrate, ■ with vegetation or □ without, in a 10 minute observation period.

DISCUSSION

Of the growing body of work on zebrafish behaviour, this is the first study to compare the behaviour of domesticated zebrafish in the laboratory with that of wild zebrafish under semi-natural conditions. The wild fish used in the field-based experiment were held in identical environmental conditions to those they experienced in the ponds from which they were collected. The period of captivity did not exceed 48 h and both feeding and spawning behaviour appeared normal throughout the course of the experiment.

Choice of oviposition site is one of the few ways in which oviparous species with no parental care can maximize offspring survival. This study demonstrates that zebrafish do not scatter their eggs haphazardly during spawning. In both the laboratory experiment and the field-based experiment there was a clear preference for a gravel substrate over silt, by both males and females (Figs. 1, 2, 4 & 5). In both studies some eggs were recovered from the floor of experimental aquaria, outside the spawning sites, though the density of eggs was always significantly greater inside the spawning sites. In the field-based study no eggs were deposited on silt in any of the replicates, despite the fact that silt is the predominant substrate in the natural habitat of zebrafish (Spence *et al.* 2006a, paper B). An adaptive explanation for this preference is provided by the enhanced survival of eggs in gravel, which was demonstrated in the laboratory incubation study (Fig. 3); eggs incubated in gravel showed significantly higher survival than in silt. This effect is probably because eggs incubated in gravel are better oxygenated than those incubated in silt, where they may face a greater risk of suffocation or infection by microorganisms (Wootton 1998).

Oxygen availability is critical for developing embryos and determines both the rate and success of development (Kamler 1992). Parental care often involves oxygenating eggs (Breder & Rosen 1966) and oxygen appears to be an important cue in oviposition decisions. In a study on embryo survival in beaugregory damselfish, artificial spawning sites with low oxygen concentration were avoided by spawning females (Payne *et al.* 2002). In the European bitterling, female choice of mussel spawning sites matches the survival of embryos (Smith *et al.* 2000), and appears to be based on the oxygen conditions inside the mussel gill (Smith *et al.* 2001a). Siltation of spawning redds is major determinant of salmonid spawning success (Chapman 1988; Rubin & Glimsäter 1996), while egg survival in species such as northern pike, *Esox lucius,* and European perch, *Perca fluviatilis,* has been shown to be lower for eggs deposited on silt than on submerged vegetation (Wright & Shoesmith 1988; Smith *et al.* 2001b).

Another advantage of gravel as a spawning substrate is that the eggs fall between the gravel and are thereby protected from disturbance or cannibalism. In this experiment the eggs were removed as soon as spawning had ceased in order to prevent cannibalism. However, in nature there is a substantial risk of egg cannibalism, or predation from species such as glassy perchlets and the introduced Nile tilapia, which are typically found in the same habitat and forage on the substrate (Spence *et al.* 2006a, paper B). Thus, the enhanced survival of eggs in gravel, demonstrated by incubating eggs under controlled laboratory conditions, probably underestimates the true fitness benefit of using gravel under natural conditions.

The laboratory choice experiment demonstrated a preference for vegetation in both substrate treatments (Fig. 1), although vegetation had no effect on egg survival (Fig. 3). While it is possible that vegetation also offers protection from cannibalism, this preference was not reflected in the field-based study (Fig. 4). Moreover, zebrafish in the field-based choice experiment did not spend any more time in vegetated than open areas of the aquarium, either during this experiment or in observations conducted outside the spawning period (Spence *et al.* 2006a, paper B). Vegetation is thought to be important to the survival of larval zebrafish; they possess attachment organs that may assist them in reaching the surface to inflate their swim bladders (Laale 1977). The ponds and lakes that zebrafish naturally inhabit often have vegetation at the margins (Spence *et al.* 2006a, paper B) and zebrafish have been observed spawning among grass stems at the flooded margins of ponds (R. Spence, pers. obs.). Water depth was not manipulated in the present study, though if zebrafish spawn preferentially at the margins of the waterbodies in which they are found, it is likely that the eggs would be deposited amongst vegetation.

Wild zebrafish displayed the same courtship and territorial behaviours during spawning as have been described in the laboratory (Spence & Smith 2005, paper D). Courtship involves males actively pursuing females, who utilize the whole of the water column, alternately swimming towards the surface and then diving steeply down to the substrate. Small groups of 3-7 fish were involved in these chases. In both the field-based study and the laboratory study, at least one territorial male was observed in each replicate.

In the laboratory choice experiment, both territorial males and females spent more time over gravel than silt substrates, though they did not spend more time over gravel with plants despite the fact that more eggs were deposited there (Fig. 2a & b). Territorial males rarely defended silt, although females did visit the silt spawning sites and spent more time over the vegetated than non-vegetated silt substrates. In the field-based experiment, territorial males defended gravel more than silt substrates but there was no overall association between spawning habitat type and the spatial distribution of females (Fig. 5a & b). This result implies that females may be visiting and assessing potential spawning sites prior to releasing eggs. Territorial males similarly may choose which oviposition sites to defend based on the substrate type, thereby maximizing the number of spawnings in which they can participate.

The field-based experiment also confirmed that spawning in zebrafish under natural conditions occurs at dawn, as has been previously supposed (Legault 1958). However, although spawning was largely limited to a short period at dawn, fish were also observed spawning during heavy rain at other times of the day. It has been noted by Breder & Rosen

(1966) that adding a dash of cold water to aquaria can encourage spawning in zebrafish. Thus, it may be that a drop in water temperature or an increase in water level may be additional cues used by zebrafish.

In conclusion, this study shows that zebrafish spawning behaviour in wild fish under semi-natural conditions is similar to that observed for domesticated strains in the laboratory. Spawning is largely confined to a brief period at dawn, although it can occur at other times and cues other than light may be involved. Female zebrafish are choosy about oviposition sites and these choices may have consequences for reproductive success. The choices of sites to defend by territorial male zebrafish also appear to relate to spawning site quality.

CHAPTER FOUR: LEARNED RESPONSES TO VISUAL SIGNALS

Paper H: The role of early learning in determining shoaling preferences based on visual cues in the zebrafish

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Authors: Rowena Spence and Carl Smith

Abstract

Social preferences for specific colour patterns learned in early development, termed imprinting, may influence social and mating decisions. Imprinting has been demonstrated in both birds and fish and has been proposed to play a role in speciation. In this study individual wild-type zebrafish were raised either with groups of the same colour pattern, an alternative colour pattern (leopard danios), or a closely related, unpatterned species, pearl danios. Individual leopard and pearl danios were similarly cross-reared with wild-type zebrafish. The study tested the prediction that fish would prefer to shoal with individuals of the colour pattern with which they had been raised, irrespective of their own appearance. Rearing condition affected shoaling preferences between wild-type zebrafish and pearl danios, with individuals of both colour patterns preferring to associate with shoals of the colour pattern with which they had been raised. The more subtle distinction between the wild-type and leopard danio colour patterns did not elicit a shoaling preference. Thus, zebrafish exhibit shoaling preferences based on visual cues, the effect being stronger when patterns are more distinct. There is a strong learned component to these preferences, although the extent to which they may influence mating decisions is unclear.

INTRODUCTION

The pigment patterns of animals are both obvious and highly variable characters that serve a variety of functions (Johnstone 1997). Pigment patterns are known to play important roles in adaptive radiation and speciation (Schluter 2000, Seehausen *et al.* 1999), and are the basis of many sexually selected traits (Andersson 1994). Colour patterns in fishes are associated with a variety of significant behavioural interactions, including shoaling, predator avoidance, territoriality, and mate choice (Guthrie & Muntz 1993; Milinski 1993; Houde 1997).

The appearance of parents and siblings that individuals are exposed to in early life can serve as a template for future social and mate choices, through the well-recognised but poorly understood behavioural mechanism termed "imprinting". The adaptive value of imprinting is thought to arise through providing a reliable mechanism for the recognition of members of the same species (Todd & Miller 1993; Irwin & Price 1999). Template formation may involve both genetically programmed and learned components and is usually restricted to a sensitive period during development (Blaustein 1983; Waldman 1988). The degree of genetic constraint governing the flexibility of template formation and extent of its persistence in later life is likely to vary according to the ecology of the species concerned (Waldman 1988).

Imprinting can promote assortative mating and thus may act as a powerful mechanism of prezygotic isolation, and thereby speciation (ten Cate & Bateson 1988; Irwin & Price 1999; Owens *et al.* 1999). Models of evolution that incorporate imprinting show that the behaviour can speed up the rate of evolutionary change by several orders of magnitude (Todd & Miller 1993; Laland 1994). Imprinting has been extensively studied in birds but has also been demonstrated in fishes and mammals (Barlow *et al.* 1990; Warburton & Lees 1996; Kendrick *et al.* 1998).

The zebrafish represents a good model for investigating the role of behavioural imprinting in speciation. There are approximately 45 danionin species worldwide (Fang 2003), many with overlapping ranges, and considerable variation in pigment patterns (Barman 1991; Talwar & Jhingran 1991). Many are closely related and able to hybridise in the laboratory. Parichy & Johnson (2001) showed that hybrids between *D. rerio* and four closely related *Danio* species all expressed pigment patterns resembling wild-type *D. rerio*. Conversely, a number of mutant *D. rerio* strains express pigment patterns which bear a close resemblance to those of other *Danio* species (Parichy & Johnson 2001).

Danionin fishes have been demonstrated to exhibit imprinting behaviour based on pigment patterns. McCann & Carlson (1982) raised striped wild-type zebrafish either with conspecifics or with a closely related unstriped species, the pearl danio (Fang 2003). When fish were later allowed to choose the colour pattern of other fish with which to associate, they preferred the colour pattern of those they had been raised with. More recently, Engeszer *et al.* (2004) replicated McCann & Carlson's results using the zebrafish pigment mutant *nacre* (Lister *et al.* 1999). The results of these studies demonstrate that *Danio* spp. pigment patterns serve as signals, and early experience with signal variation may influence future social preferences. Thus, a single locus pigment mutation can potentially cause dramatic changes to the social associations an individual makes. For fishes the most important social interactions are in shoaling and spawning. Since mating is more likely to take place with others in the same social unit, it has been proposed that shoaling preferences may promote assortative mating. Consequently, even without strong mate preferences in relation to colour pattern, shoaling preferences could promote reproductive isolation and thereby genetic divergence among populations.

A role for imprinting in mate choice and speciation thus assumes naturally occurring pigment mutation can affect shoaling preferences within species. Such mutations are likely to differ less strongly from the wild-type pattern than the contrast between stripes and lack of stripes typified by imprinting on pearl danios or *nacre*. The present study used imprinting to compare shoaling preferences between wild-type zebrafish leopard danios, as well as between wild-type zebrafish and pearl danios. Rosenthal & Ryan (2005) tested the shoaling preferences of several *Danio* colour patterns with computer animated stimulus shoals of either striped or unstriped fish. While striped individuals had a strong preference for stripes and unstriped individuals a strong preference for no stripes, leopard danios, which have melanophores arranged in spots rather than stripes, showed no significant preference. The present study tested the prediction that rearing conditions can influence subsequent social preferences for different colour patterns. It was predicted that fish would prefer to shoal with the colour pattern with which they had been raised, irrespective of their own appearance. It was further predicted that the preference would be stronger between striped, wild-type zebrafish and unstriped pearl danios than between wild-type zebrafish and spotted leopard danios.

METHODS

Imprinting procedure

Experimental fish were bred in the laboratory from stock obtained from a commercial supplier. The stock used comprised both wild-type zebrafish, leopard danios and pearl danios. They were housed in an environmentally controlled room at 24 °C with a 14:10 h light: dark cycle (0800 AM-1000 PM). Larvae were reared in plastic aquaria (34 x 20 x 20 cm), initially in 20 mm of water, the depth being increased as the larvae grew. They were fed three times a day with infusoria and powdered fry food (Cyprico Crumble Excellent, 200-300 μm). Water quality was maintained with daily water changes. Embryos of each colour pattern were kept in separately labelled aquaria for the first two weeks following hatching. All three colour patterns develop from identical larval melanophore patterns which only start to differentiate after 19 days (McClure 1999), early adult pigment pattern being formed by around 28 days (Parichy & Johnson 2001). After two weeks fish were isolated in groups of 10 for imprinting.

Fish were imprinted either on their own colour pattern (control) or on an alternative colour pattern (cross-reared). Cross-reared fish were raised singly with nine fish of the alternative colour pattern. Control fish were also split into groups of 10. There were seven treatments in total: control wild-type zebrafish (WW); control leopard danios (LL); control pearl danios (PP); single wildtype zebrafish cross-reared with nine leopard danios (WL); single leopard danios cross-reared with nine wild-type zebrafish (LW); single wildtype zebrafish cross-reared with nine pearl danios (WP); and single pearl danios cross-reared with nine wild-type zebrafish (PW). At six weeks of age the groups were transferred to 20 l glass aquaria (40 x 30 x 25 cm) with water on a recirculating system at 27 °C. Plastic plants were placed in each aquarium but no substrate material was used. They were fed three times each day with a mixture of frozen bloodworm, frozen brineshrimp and high protein commercial ornamental fish feed pellets (Cyprico Crumble Excellent).

Shoaling preference

Shoaling preference was tested in February 2005, when the fish were 19 weeks old and before sexual maturity (Fig. 1). Preferences were tested between wild-type zebrafish and leopard danios using six replicates each of WW, WL, LL and 10 of LW. Preferences were then tested between wild-type zebrafish and pearl danios using a further six replicates of WW, and six each of WP, PP and PW, a total of 52 fish. The mean ± SD SL of fish used in the study was 25.7 ± 3.69 (wild-type zebrafish), 24.7 ± 3.84 (leopard danios) and 23.8 ± 2.33 (pearl danios). Tests were conducted in an experimental glass aquarium (120 x 43 x 34 cm) with a smaller stimulus aquarium (33 x 20 x 20 cm) placed sideways at each end, making three compartments. Experimental and stimulus fish could see each other but there was no olfactory contact between them. Right and left preference areas of the experimental aquarium were

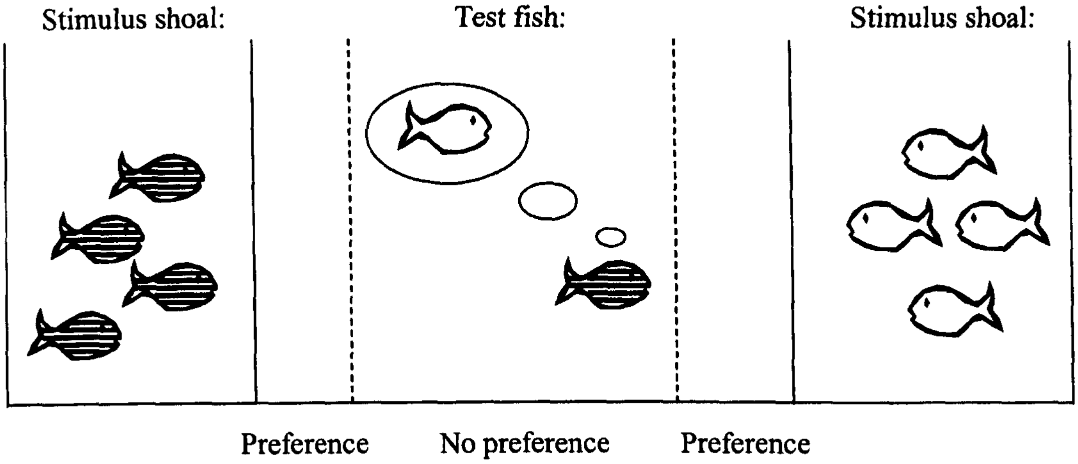

Figure 1. Schematic representation of test for shoaling preference, showing a test fish of the Treatment 'wild-type zebrafish cross-reared with pearl danios', and stimulus shoals of wild-type zebrafish and pearl danios.

marked on its exterior, 90 mm in from the edge of each stimulus aquarium, giving three areas: right, left and centre. This distance was chosen to represent approximately three body lengths, based on the definition of shoaling behaviour as fish behaving together within four body lengths (Pitcher & Parrish 1993). The aquaria were filled with water to a depth of 25 cm and heated to 27°C.

Stimulus fish of the same age and matched in size to the test fish were placed in the stimulus aquaria. Stimulus fish comprised six wild-type zebrafish at one end and six of the alternative colour pattern at the opposite end, according to a random protocol. Shoals of as few as four fish exhibit shoaling behaviour indistinguishable from those in larger groups (Breder & Halpern 1946) and, under natural conditions, shoal sizes vary between 2-30 fish (Spence *et al.* 2006a, paper B). Stimulus fish were taken from mixed-colour pattern shoals in order to ensure that they did not preferentially respond to the colour pattern of the test fish. Stimulus fish were allowed 30 min to acclimatize to the aquaria, after which a randomly selected test fish was placed in the middle of the central compartment. The test fish was observed until it had attempted to interact with both the stimulus shoals. During the following 5 min, the amount of time the test fish spent in each of the three areas (left, right and centre) of the aquarium was recorded. A subset of 18 fish was tested 3 times, on different days, in order to control for consistency of preference. Stimulus shoals were replaced with different individuals between trials. At the end of the experiment all experimental fish were retained in the aquarium.

Data analysis

All data were tested for normality using a Kolmogorov-Smirnov test and for equality of variance using Bartlett's test. All proportional data were arcsine transformed before analysis (Zar 1996). A two-way ANOVA was used to identify the effects of rearing condition and colour pattern on the proportion of time spent in association with like colour pattern. A nested, one-way ANOVA was used to test for consistency of preference between trials.

RESULTS

In tests of shoaling preference among wild-type zebrafish and leopard danios there were no significant effects of either colour pattern (two-way ANOVA: $F_{1,24} = 0.33$, $P = 0.568$) or rearing condition (two-way ANOVA: $F_{1,24} = 0.07$, $P = 0.794$) on the proportion of time spent in association with like colour pattern, and there was no interaction (two-way ANOVA: $F_{1,24} = 0.01$, $P = 0.955$; Fig. 2). Among wild-type zebrafish and pearl danios, rearing condition had a significant effect on the proportion of time spent in association with like colour pattern (two-way ANOVA: $F_{1,20} = 41.50$, $P < 0.001$). There was no effect of species (two-way ANOVA: $F_{1,20} = 2.99$, $P = 0.099$) but there was a significant interaction (two-way ANOVA: $F_{1,20} = 8.01$, $P = 0.010$), the effect of rearing condition being stronger among pearl danios (Fig.3). There was no significant difference in preference among trials for the subset of fish tested for consistency (one-way nested ANOVA: $F_{2,51} = 1.891$, $P = 0.161$).

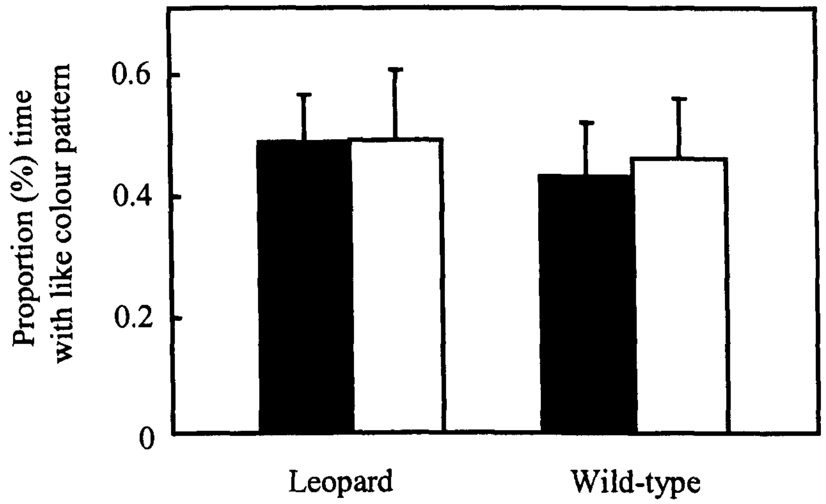

Figure 2. Mean + SE proportion (%) of time spent by test fish (■ cross-reared and □ control wild-type and leopard *D. rerio*) in association with the stimulus shoal of like colour pattern.

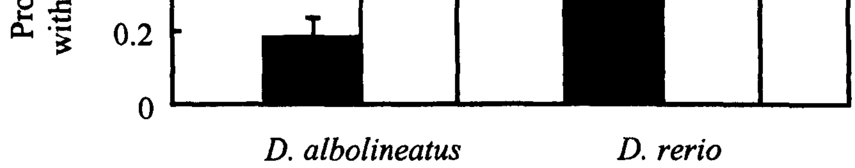

Figure 3. Mean + SE proportion (%) of time spent by test fish (■ cross-reared and □ control wild-type *D. rerio* and *D. albolineatus*) in association with the stimulus shoal of like colour pattern.

DISCUSSION

This study used danionin fishes of three different colour pattern variations, striped, spotted and plain, to test the prediction that imprinting may play a role in determining subsequent social interactions. Rearing condition significantly affected shoaling preferences between wild-type zebrafish and pearl danios; test individuals preferred to associate with shoals of the colour pattern with which they were raised, irrespective of their own colour pattern. This finding broadly corresponds with that of McCann & Carlson (1982), except that they found the effect was stronger for wild-type zebrafish than pearl danios. There was no significant preference between the wild-type zebrafish and leopard danios for either colour pattern under either rearing condition.

The most parsimonious explanation for these results is that the difference between the striped wild-type and spotted leopard colour patterns was insufficient to elicit a preference, compared with the difference between a striped pattern and an absence of stripes exemplified by wild-type zebrafish and pearl danios respectively. An alternative explanation is that imprinting operates between but not within species. However, this explanation is less likely; the use of cross-reared fish demonstrates that colour pattern preferences are learned rather than innate. In addition, similar preferences have been demonstrated using wild-type zebrafish and pigment mutants which lack stripes (Engeszer *et al.* 2004). While there is a possibility that results obtained using mutants may be due to pleiotropic effects of the mutation, the two studies together suggest that the effect is most likely to be due to the presence or absence of stripes. The findings of this study are also consistent with Rosenthal & Ryan's (2005) study which suggested that stripes are a shoaling cue among *Danio* spp. fishes.

While it is possible that imprinting may have occurred during the first two weeks of the study, before cross-reared fish were placed with their foster groups, this does not explain the results obtained. The use of self-imprinted controls showed that, irrespective of rearing condition, there was assortative shoaling between wild-type zebrafish and pearl danios, but not between wild-types and leopard danios; the same imprinting protocol was used for all three colour patterns. As embryos were imprinted on others of the same age, not on parental colour patterns, this early period of exposure would not have affected subsequent imprinting on colour patterns. Imprinting in this sense is not comparable with that demonstrated in precocial bird species which become imprinted immediately upon hatching (Bolhuis 1991).

Thus, it appears that zebrafish do exhibit shoaling preferences based on visual cues and that there is a strong learned component to these preferences. Fish are capable of both individual recognition based on prior experience and recognition of unfamiliar individuals based on phenotype matching (Griffiths 2003; Ward & Hart 2003). Familiarity has been shown to be an important mechanism governing social interactions both within and among fish species (Ward *et al.* 2003). The present study suggests that in the absence of familiarity, phenotype matching mediates shoaling decisions, and that there is plasticity in the development of the template. Warburton and Lees (1996) similarly showed that guppies raised with swordtails, *Xiphophorus helleri*, preferred to associate with swordtails rather than conspecifics.

Among fishes, there is likely to be stronger selection pressure for the development of recognition ability in shoaling than solitary species. Brown & Colgan (1986) found that familiarity was important in social interactions in bluegill sunfish, whereas in rock bass, *Ambloplites rupestris,* social experience was not a necessary condition for species recognition as individuals reared in isolation preferred to associate with conspecifics. They suggest that species recognition may be based on a closed genetic program in solitary species and an open programme in shoaling species. The present study indicates that an open program operates in *D. rerio*. Moreover, Engeszer *et al.* (2004) found that zebrafish reared in isolation did not show a shoaling preference between striped and unstriped shoalmates.

Whether shoaling preferences resulting from cross-rearing are likely to translate into mating preferences is unclear, although pilot studies indicate that they do not (R. Spence, unpublished data). The relationship between shoaling preferences and mating preferences is not straightforward. Mate choice in species such as guppies and three-spined sticklebacks is strongly influenced by colour pattern although shoaling preferences are not (Bakker 1993; Houde 1997). Further, both male and female guppies have been shown to prefer unfamiliar mates to mates from the same shoal (Hughes *et al.* 1999; Kelley *et al.* 1999). Even in birds, there is considerable variation among species in the role of sexual imprinting in mate choice (Slagsvold *et al.* 2002).

The extent to which the shoaling preferences demonstrated in this study would apply in nature is also unclear. Although many species show active shoaling preferences for conspecifics, mixed species shoals are common in nature and shoal fidelity among shallow freshwater communities is often low (Krause & Hoare 2003). Zebrafish and pearl danios shoal together in the laboratory while in nature zebrafish often shoal with the closely related flying barb (Spence *et al.* 2006a, paper B). Moreover, McCann & Carlson (1982) found that the effects of cross-rearing on zebrafish and pearl danios were no longer significant when fish were re-tested after four weeks of housing with fish of the same colour pattern. Nevertheless, species recognition is a pre-requisite for mate choice and is thought to be based on social learning. Importantly, individual recognition in fish is often mediated by olfactory cues (Ward & Hart 2003). This factor was not manipulated in the present study although both kin and individual recognition based on olfactory cues has been demonstrated in zebrafish (Mann *et al.* 2003, Gerlach & Lysiak 2006). The opportunity for imprinting may be greater in species with extended parental care, such as guarder and bearer cichlid species, where fostering of young has been reported both within and among species (Keenleyside 1991). There is some evidence that early chemical and visual cues can have lasting effects on social responses in such species.

In conclusion, zebrafish exhibit shoaling preferences based on visual cues, the effect being stronger when colour patterns are more divergent. There is a strong learned component to these preferences, suggesting a mechanism of phenotype matching based on early experience. The extent to which visual cues alone are sufficient to influence mating decisions is unclear.

Paper I: Innate and learned colour preference in the zebrafish

This paper has been submitted to: *Frontiers in Zoology*

Authors: Rowena Spence and Carl Smith

Abstract

Innate preferences for specific colours have been demonstrated in insects, birds and fish, both in the context of foraging and mate choice. Learning can also play a role in determining preferences, and observed preferences may be the result of an interaction between innate and learned behaviour. This study tested for a visual receiver bias in the zebrafish and investigated the role of learning in shaping colour preferences. Groups of fish were raised on diets consisting solely of red, blue, green or white food. When their colour preferences were subsequently tested in a foraging context, each group responded most strongly to red, irrespective of the colour of food with which they had been conditioned. However, conditioning did significantly influence colour preferences. The observed receiver bias towards red may have evolved as a function of the nature of the transmission environment zebrafish inhabit, in combination with an adaptive preference for carotenoid compounds in their diet.

INTRODUCTION

Signals play an important role in animal ecology, including foraging, predator avoidance, species recognition and mate choice (Maynard Smith & Harper 2003). Signal efficacy, the extent to which a signal affects the behaviour of the receiver, depends not only on the physical constraints of the transmission environment and the sensory system of the receiver but also on the receiver's cognitive capabilities, which determine how the signal is interpreted (Chittka & Menzel 1992; Endler 2000). A receiver bias arises when the characteristics of an organism's sensory system or psychology cause it to respond more strongly to certain signals. Biases can exist at any level along the signal reception and processing continuum from stimulation of a primary sensory receptor to synthesis at higher levels of integration, including learning, memory and decision making (Basolo 2000).

Receiver biases for certain colours have been demonstrated in various taxa, in the context of both foraging and mate choice (Lunau & Maier 1985; Milinski & Bakker 1990; Schuler & Roper 1992; Houde 1997). In some cases, ecological interpretations can be offered for innate colour preferences. For instance, there is a correlation between the innate colour preferences of honeybees and the nectar production of melittophilous flowers (Giurfa *et al.* 1995), while birds tend to show innate avoidance responses to colours associated with aposematic prey (Guilford 1990). Experience is also an important factor in determining preferences, and the interaction between innate and learned preferences has been the focus of considerable research. Innate preferences affect the speed of associative learning (Menzel 1985), and training can interfere with innate preferences (Kelber 1996; Weiss 1997; Gumbert 2000; Honkavaara *et al.* 2004).

One ecological interpretation for colour preferences in mate choice is offered by the 'pre-existing bias' theory of sexual selection (Endler & Basolo 1998; Ryan 1998). For example, the preference of female guppies for orange colouration in males may have originated as a pleiotropic side effect of foraging behaviour (Rodd *et al.* 2002). In the three-spined stickleback, females show a strong preference for males with red nuptial colouration (Wootton 1976). This preference by females also appears to have evolved in a foraging context; an experimental study combined with phylogenetic analysis demonstrated that the bias emerged prior to the evolution of the red signal (Smith *et al.* 2004b). Nine-spined sticklebacks, *Pungitius pungitius,* which lack red nuptial colouration, also exhibited a preference for red food items (Smith *et al.* 2004). A red preference has also been demonstrated in a foraging context in European bitterling (C. Smith unpublished data), and the poeciliid *Brachyraphis episcopi* (Brown & Braithwaite 2005). The explanation for the observed preference for red food items in the diet of sticklebacks (Ibrahim & Huntingford 1989) may relate to a requirement for carotenoids (Ringelberg 1980). The red pigment of three-spined sticklebacks is principally derived from the carotenoid pigments astaxanthin and tunaxanthin/lutein (Wedekind *et al.* 1998). The inclusion of carotenoids in the diet is thought to confer fitness benefits (Olsen & Owens 1998). Since carotenoids cannot be synthesized *de novo* by animals, zooplankton are the main dietary source for many fishes (Rønnestad *et al.* 1998).

The present study used an experimental approach to test for a receiver bias in a foraging context and explore the role of learning in shaping colour preferences in the zebrafish. The zebrafish displays neither red colouration nor strong sexual dimorphism. In common with that of many vertebrates, zebrafish colour vision is tetrachromatic, with red, green, blue and UV-sensitive cones (Robinson *et al.* 1993). Groups of zebrafish were reared on different coloured foods and their foraging preferences were subsequently tested. Given the importance of carotenoids in the diet of fishes, and because zooplankton are known to form the main component of the natural diet of zebrafish (Dutta 1993; Spence *et al.* in press, paper C) it was predicted that there would be an innate receiver bias towards red. In addition, the study tested whether the foraging responses of zebrafish could be modified by associative learning.

METHODS

Zebrafish were bred in the laboratory from stock obtained from a commercial supplier. They were housed in an environmentally controlled room with a 14:10 h light: dark cycle (0800 AM-1000 PM). Four groups of larvae were reared in plastic aquaria (34 x 20 x 20 cm), initially in 20 mm of water, on a recirculating system at 25°C, water depth being increased as the larvae grew. At 6 weeks of age fish were transferred to sixteen 20 l glass aquaria (40 x 25 x 30 cm). For the first 14 days of development, larvae were fed three times each day with infusoria. Thereafter they were fed with high protein powdered fry food (Cyprico Crumble Excellent, 200-300 μm), coloured with either red, blue, green or white edible powdered food dye. There were four groups of fish, each exposed to one of the four diet colour treatments. Dry food was supplemented twice each week with fresh chopped white worms, *Enchytraeus* spp., coloured with the same dye. Reflectance spectra of the different colours of food were measured using a spectrometer (Ocean Optics, Dunedin, FL, S2000 with a deuterium/halogen light source), for methods, see Chittka & Kevan (2005). The reflectance spectra of differently coloured food are shown in Figure 1. Each group was visually isolated from adjacent aquaria throughout rearing until the end of the experiment. At the end of the study all fish were returned to a normal diet and retained in the aquarium.

Colour preference was tested in October 2005, when the fish were 16 weeks old and not yet sexually mature. Mean ± SD SL of fish was 21.3 ± 2.11 mm (red), 22.0 ± 1.51 mm (blue), 22.1 ± 1.51 mm (green) and 22.4 ± 1.87 (white). Tests were conducted in glass aquaria identical to those in which the fish were reared, illuminated with a 40W daylight bulb in addition to overhead fluorescent lighting. Fish were tested in pairs; solitary zebrafish sometimes show signs of distress when isolated. They were transferred to test aquaria in the evening and allowed to acclimatise overnight, with tests conducted the following morning. The pairs were fed to satiation after transfer to test aquaria in order to standardise their motivation to feed. During tests, the fish were consecutively presented with five differently coloured strips of plastic (red, blue, green, black, white) with a mean diameter of 1.4 ± 0.07 mm and a mean length of 2.8 ± 0.25 mm, in a randomly determined order that varied among pairs and treatments. The reflectance of the coloured plastic strips was measured using a spectrometer (Ocean Optics, Dunedin, FL, S2000 with a deuterium/halogen light source), for methods, see Chittka and Kevan (2005). Since the measurement area (diameter of 5 mm) exceeded the width of the plastic strips, several strips were glued side by side on black electrical isolation tape (see Chittka and Kevan 2005). Reflectance spectra of the plastic strips are shown in Figure 2. The strips of plastic were tied to a *ca.* 150 mm length of 0.1 mm nylon thread, and when a plastic strip was presented to a pair of fish it was suspended, stationary, in the water column in the upper half of the test aquarium.

Each pair of fish was observed for 90 s following the introduction of the plastic strip and the total number of bites directed at the strip by both fish was recorded. Fish were used in trials only once, each pair representing a single replicate.

Data analysis

All data were tested for normality using a Kolmogorov-Smirnov test and for equality of variance using Bartlett's test. Data were square-root transformed and were analysed using a General Linear Model with colour of strips and colour on which pairs had been conditioned as independent variables and bite rate as the dependent variable. Order of presentation of plastic strips was an additional independent variable.

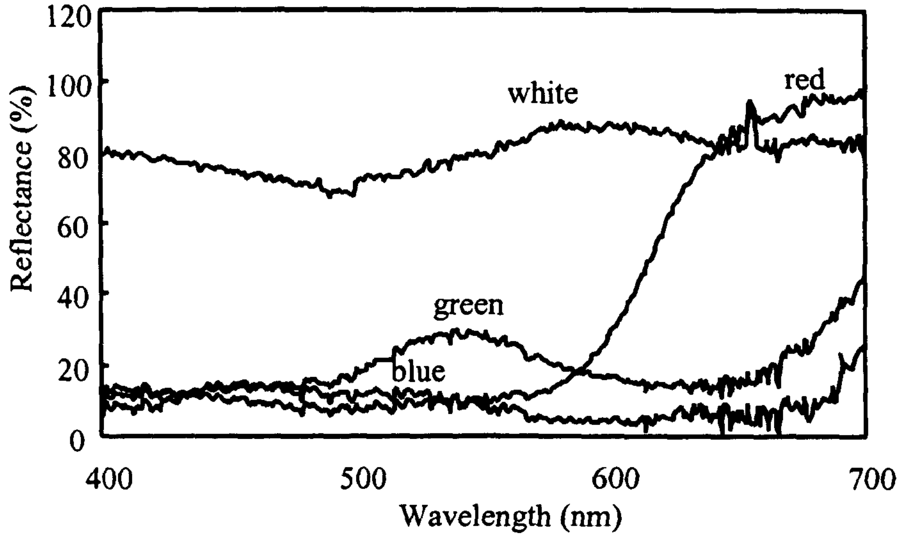

Figure 1. Reflectance spectra of the different coloured food used to condition zebrafish.

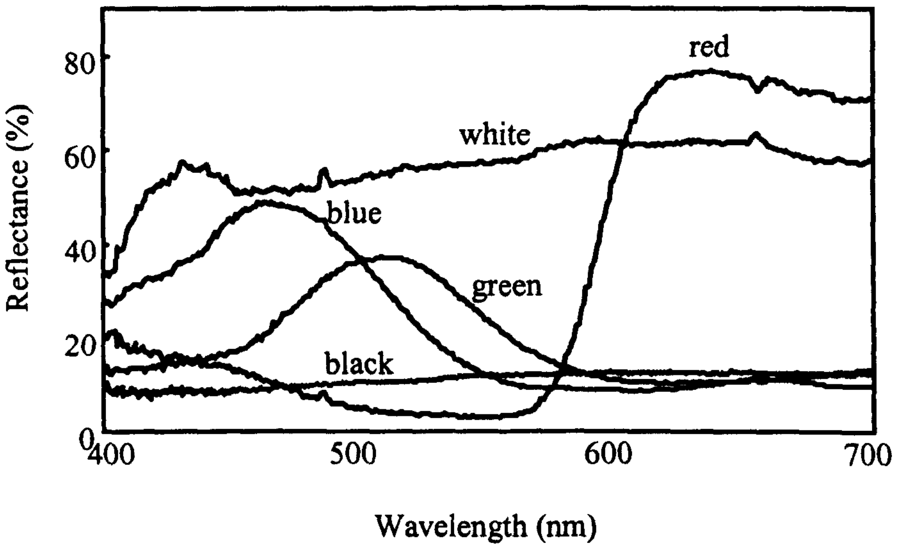

Figure 2. Reflectance spectra of the coloured plastic strips used in the experiment.

RESULTS

The rate of biting at coloured strips was tested for 10 pairs each of fish conditioned on red, blue or green food, and five pairs conditioned on white food. Bite rate was significantly affected by order of presentation (GLM: $F_{4,151} = 12.07$, $P < 0.001$), and this variable was used as a block effect in the analysis. The rate of biting was significantly different among coloured strips (GLM: $F_{4,151} = 13.95$, $P < 0.001$, Fig. 3). The highest rate among all colours, irrespective of conditioned colour treatment, was directed at red strips (Tukey's pairwise comparison, $P < 0.001$). In addition, there was a significant effect of colour conditioning (GLM: $F_{3,151} = 3.39$, $P = 0.020$), with a significantly higher rate of biting directed at strips which matched the conditioned colour than those which did not (Tukey's pairwise comparison, $P = 0.01$). There was no significant interaction between test colour and colour on which fish were conditioned (GLM: $F_{12,151} = 0.98$, $P = 0.466$).

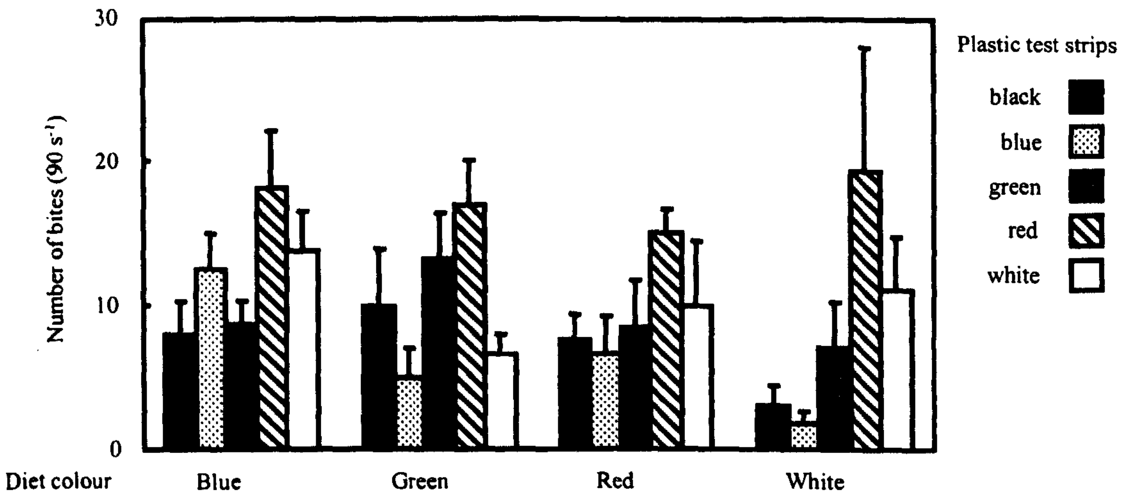

Figure 3. Mean + SE bite rate (bites 90 s-1) at coloured plastic strips (black, blue, green, red, white) by groups of zebrafish conditioned on different coloured food (blue, green, red, white).

DISCUSSION

Zebrafish showed a highly significant innate preference for biting at red plastic strips, irrespective of the colour of food on which they had been conditioned. However, conditioning did influence preferences for colours other than red. The behaviour of the fish when biting the plastic strips was consistent with feeding behaviour; they swam straight at the strips and bit them immediately. When inspecting novel objects, zebrafish approach them cautiously (Wright *et al.* 2006a), while aggressive behaviour is always accompanied by displays (Spence & Smith 2005, paper D).

On the basis of the match between the reflectance spectra of the coloured plastic strips used in our experiment and zebrafish photopigment spectral sensitivity, it can be inferred that the test fish were able to discriminate equally between the four test colours. Zebrafish have four types of visual pigment, with peak absorbance in ultraviolet, (362 nm) (Robinson *et al.* 1993), blue (415 nm), green (480 nm) and red (570 nm) (Hughes *et al.* 1998). Spectral sensitivity is greatest at shorter wavelengths (340-425 nm) (Cameron 2002). The zebrafish retina contains four cone-processing channels, two of which are non-opponent (ultraviolet and short) and two that use wavelength opponent processing (long-medium and medium-short) (Cameron 2002). Thus, the zebrafish visual system appears to be adapted to detect luminance contrast at shorter wavelengths and chromatic contrast at medium and longer wavelengths. This dual adaptation can be understood in the context of zebrafish ecology.

Under natural conditions, zebrafish are zooplanktivores (Dutta 1993) and inhabit shallow ponds and lakes in the flood plains of sub-tropical rivers (Sterba 1962; Talwar & Jhingran 1991; Spence *et al.* 2006a, paper B) where the concentration of chlorophyll and dissolved organic compounds is high. Sensitivity to luminance contrast at short wavelengths (which penetrate water more readily than longer wavelengths) enhances the detection of contrast against a bright background of down-welling light (Losey *et al.* 1999). Browman *et al.* (1994)

showed that prey detection by zooplanktivorous fish was enhanced in ultraviolet light conditions. Unlike some teleost fishes, zebrafish retain ultraviolet vision throughout life. However, at longer wavelengths sensitivity appears to be greater to chromatic contrast. Discrimination in these wavelengths is important in the aquatic environment where the spectrum of down-welling light shifts and narrows with increasing depth and the presence of dissolved organic compounds (Lythgoe 1979).

The ecological basis of the red preference may relate to the natural diet of zebrafish, which is rich in microcrustaceans, such as copepods, cladocerans and ostracods (Dutta 1993; Spence *et al.* in press, paper C). Many fishes are primarily visual predators (Guthrie & Muntz 1993) and there is abundant evidence that the degree of red pigmentation in zooplankton increases its risk of predation (Hairston 1979; Bakker *et al.* 1997; Hansson 2000; Van der Veen, 2005). Utne-Palm (1999) showed that predator reaction distance to copepods increased as a function of prey contrast, which in turn increased with wavelength; the degree of contrast being greater for red than transparent copepods. In addition to ease of detectability, the potential benefits of a diet rich in carotenoids (Olsen & Owens 1998) may favour the evolution of a preference for red in the diet of fishes. Ibrahim & Huntingford (1989) showed that sticklebacks selected prey primarily on the basis of visual cues, with red colouration a better predictor of diet choice than profitability. Thus, red colouration may function as a proximate cue associated with profitability.

The UV reflectance of food and test colours were not measured, though these may have differed. One possibility is that zebrafish may show an innate preference for high UV reflectance, which could account for the apparent innate preference for red in our study (if the red plastic strips had a higher UV reflectance than the other colours). However, the white plastic strips would be most likely to have the highest UV reflectance and these were not preferred by fish. Thus, while the experimental design does not exclude the possibility of an innate red and UV preference in zebrafish, further research will be needed to determine what the role of UV wavelengths are, if any, in zebrafish innate diet preferences.

The study detected a significant effect of conditioning; fish responded significantly more strongly to the plastic strips which equated to the colour of food on which they had been conditioned than those which did not (Fig. 3) The reflectance spectra of the food dye on which fish were conditioned and that of the plastic strips with which they were tested were not identical (Figs. 1 and 2). However, differences among different test colours were greater than those between food and test colours. Red and green food and test colours all showed distinct peaks, with those of the foods being at slightly longer wavelengths than those of the test strips. The peak reflectance of red food was between 650-700 nm, whereas that of the red test strip was between 600-700 nm. The peak of reflectance of green food was between 500-600 nm, and that of the green test strip was between 450-550 nm. White test and food colours reflected across the entire spectrum. Blue food colour had no distinct peak, its reflectance being more similar to the black than blue test strips. However, reflectance is not the sole determinant of how hue is perceived and evidence suggests that human and fish perception of hue are not dissimilar. Studies of stickleback colour vision have demonstrated that human rankings of the degree of male red nuptial colouration are consistent with those that would be predicted from modelling stickleback colour vision (Rowe *et al.* 2006). Moreover, conditioned colour did influence responses to test colours, although this effect may have been stronger had the conditioned and test colours been identical.

Although learning influenced colour preferences in our study, the innate bias towards red appears to be relatively inflexible. This has implications for the design of cognitive behavioural studies involving colour discrimination; learning is liable to be faster when red is used as the positive stimulus (Brown & Braithwaite 2005). In contrast, in bees innate colour preferences appear to have a minor role in foraging (Giurfa *et al.* 1995; Gumbert 2000). In birds the evidence is more ambiguous; responses vary with species (Honkavaara *et al.* 2004), circumstances of presentation (Roper & Marples 1996; Gamberale-Stille & Tullberg 2001), and the involvement of other sensory modalities such as odour (Rowe & Skelhorn 2005). The strength of the red preference measured in this study could be interpreted as lending support to the receiver bias model of signal evolution. Much of the work in this area has concentrated on sexual signalling, using comparative methods to show biases which pre-date the evolution of a signal, although Jansson & Enquist (2003) used discrimination training in birds to demonstrate a bias towards more colourful signals. Male zebrafish do not utilise red as a nuptial colouration in the way that three-spined sticklebacks do. However, the opportunity for sexual selection in zebrafish appears to be weak (Spence *et al.* 2006b, paper E), making the evolution of sexually selected traits in zebrafish unlikely.

Colour conditioning in this study only commenced when the fish were two weeks old, by which time all the neural circuitry required for colour vision would have been fully developed. Thus the innate and learned responses we detected were not due to physical modification of neuronal inputs during development. Variation in colour preferences may provide a valuable model for studying the developmental genetics of zebrafish vision, as a large number of mutations affecting vision are available in this species (Baier *et al.* 1996; Fadool *et al.* 1997; Neuhauss *et al.* 1999).

In summary, the results of this study suggest that zebrafish have an innate bias towards the colour red, which is modified, but not superseded, by learning. This bias may have evolved as a function of the nature of the transmission environment zebrafish inhabit, in combination with an adaptive preference for critical compounds in their diet.

CHAPTER FIVE: DISCUSSION

The aim of the project was to develop a more complete understanding of the natural ecology and behaviour of zebrafish. Chapter one reviews the literature, including areas beyond the scope of the current project, such as taxonomy and the genetic basis of behaviour, as well as introducing the experimental work presented in subsequent chapters. Chapter two presents data on the basic ecology of zebrafish, based on sampling in Bangladesh. No systematic fieldwork has previously been conducted on this species, although observations by taxonomists are largely in accordance with the habitat parameters reported in paper B. Zebrafish appear to be primarily a floodplain species, inhabiting shallow ponds and ditches or the slower reaches of streams. They are an abundant species and are among the smallest fish species in the assemblage. Their diet, based on gut content analysis, consists primarily of insects, (of both terrestrial and aquatic origin) and zooplankton, as well as inorganic material. These results indicate that they feed throughout the water column, consistent with observations of their vertical distribution, and the finding that they tend to be confined to the shallow margins of waterbodies.

The zebrafish is known for its rapid development but there have hitherto been no growth studies conducted on wild fish. The length-frequency analysis reported in paper B indicates that the most rapid growth takes place in the first three months, and slows down thereafter, ceasing by about 18 months. Under natural conditions the zebrafish is an annual species and recruitment is linked to the monsoon, which is also the period of the year with the highest temperatures. However, breeding may be dependent on food availability rather than season, as gravid females were found in Bangladesh in winter and zebrafish breed all year round in the laboratory.

Due to the turbid conditions during the spawning season it was not possible to conduct observations under natural conditions, although the experiments reported in papers B and G using large, outdoor aquaria confirm that the behaviour of wild-caught zebrafish is comparable to that of laboratory strains. Further field research on zebrafish should involve identifying sites suitable for field experiments. For the zebrafish to be more widely adopted as a model by the behavioural ecology community there is a need for more behavioural and field-based studies in order to catalogue natural variation in morphological, physiological and behavioural traits. This should include comparing the behaviour of zebrafish from a number of populations with different environmental parameters and predator regimes, as has been done with guppies (Magurran 2005).

The experimental work on reproductive behaviour reported in chapter three was conducted primarily on domesticated strains, although the field experiment reported in paper G confirms that the mating behaviours described are broadly applicable in nature. Zebrafish have hitherto been characterised as group spawners and egg scatterers. However, the experiments outlined in papers D-G show that there is evidence that the mating system is influenced by both male-male competition and female mate preferences. Further, competition for high quality sites for oviposition may be a key feature of mating behaviour in nature. The role of olfactory cues in reproductive or social behaviour was not investigated. However, given the role of pheromones in zebrafish reproduction and evidence from other published behavioural studies (Gerlach 2006; Gerlach & Lysiak 2006), this might be a fruitful avenue for further research. The zebrafish may particularly be a suitable model for studying the role of MHC in mate choice. However, the zebrafish has little to offer as a model for sexual selection compared to other fish behavioural models such as guppies, sticklebacks or bitterling. The opportunity for sexual selection appears to be weak in zebrafish, as might be predicted from their lack of marked sexual dimorphism. Despite the existence of two distinct male mating tactics, territorial males only achieved superior reproductive success at low densities, and subordinate males appear to be able to fertilise a significant proportion of eggs, at least in the experimental design used here.

The experiments described in chapter four suggest that the zebrafish may have more potential as a model for studies of cognitive behaviour. Paper H shows that visual cues are important in species recognition; the underlying mechanism appears to be phenotype matching based on early social experience. An obvious extension of this study would be to examine the effects of cross-rearing on mate choice. Given the availability of zebrafish pigment mutants and their striking similarity to closely related *Danio* species, the zebrafish may be suitable as a model to elucidate mechanisms of evolutionary change and speciation.

While learned behaviour in a social context was tackled in paper H, in paper I the way in which learning shapes colour preferences in a foraging context was addressed. Together, these studies confirm that conditioning can have a significant impact on zebrafish cognitive behaviour. The zebrafish visual system is well understood and a number of visual mutants are available, which could be utilised in further studies on variation in colour preferences. The fact that zebrafish respond readily to different colours suggests that they may be suitable for studies on non-spatial learning, while other published studies show that it can be used to study spatial learning (Bilotta 2005; Colwill 2005; Williams 2002). As a shoaling species, zebrafish may also be suitable for studies of social learning (Suboski *et al.* 1990). Given our detailed understanding of zebrafish development, they would be an excellent model for studying the development of cognitive processes.

The greatest advantage of the zebrafish as a model system comes from its well characterised genetics, genetic and developmental techniques, and the availability of mutants. Thus, any behavioural studies on zebrafish should attempt to capitalise on these resources, by linking behaviour to gene expression. Pursuing this goal could either involve using known mutants in behavioural studies or comparing the behaviour of different strains of zebrafish.

Zebrafish also prove a tractable species for behavioural experiments, readily acclimatising to new environments, being constantly active and little disturbed by the presence of observers. The disadvantage of zebrafish as a behaviour genetics model is that they display limited behavioural syndromes compared to other well-studied species, such as three-spined sticklebacks and guppies. Behaviours which might be suitable for gene expression studies include dominance and territoriality, as well as the cognitive studies mentioned above.

In summary, this project has collected data on the natural ecology of zebrafish, including habitat preferences, diet, growth and recruitment. The zebrafish mating system has been described, including male mating tactics and female preferences for both mates and oviposition sites, and genetic data used to quantify the opportunity for sexual selection. Finally, zebrafish have been used to study learning in social and foraging contexts. These studies together provide a basis for further studies aimed at linking zebrafish behaviour and gene expression.

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