Reproductive chemical cues in two freshwater fishes: topmouth gudgeon *Pseudorasbora parva* (Temminck and Schlegel) and sunbleak *Leucaspius delineatus* (Heckel)

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SUMMARY

The use of reproductive chemical cues is widespread amongst fishes. However, the most understood sex pheromone systems derive from species that employ a scramble spawning reproductive strategy. This thesis investigated for the first time the use of reproductive chemical communication in topmouth gudgeon *Pseudorasbora parva* (Temminck & Schlegel) and sunbleak *Leucaspius delineatus* (Heckel) that use two different forms of a male nest guarding reproductive strategy. In topmouth gudgeon, approximately a third of reproductive females adopted a body posture in response to reproductive male conditioned water advertising high receptivity to potential mates. Electro-Olfactory Gram recordings of reproductive male and female topmouth gudgeon revealed a high magnitude response to reproductive male and female odours. In addition, both topmouth gudgeon and sunbleak reproductive females responded to chemicals cues derived from conspecific reproductive males by an increase in swimming behaviour. In contrast to male topmouth gudgeon, reproductive male sunbleak responded to chemical cues from reproductive conspecific males and females. Active compounds were isolated from reproductive male topmouth gudgeon conditioned water by two different methods; solid phase extraction (C-18 cartridges) and using a freeze drier. The eluate was subsequently separated using High Performance Liquid Chromatography into retention time fractions. An active fraction was identified using a bioassay guided separation. Nuclear Magnetic Resonance analysis showed that compounds were present in the active fraction. Chemical interaction between topmouth gudgeon (invasive to Europe) and sunbleak (native to Europe) was also investigated. Behaviour responses in the two species were asymmetrical; topmouth gudgeon did not respond to sunbleak chemical cues. In contrast, both reproductive female and male sunbleak responded to topmouth gudgeon chemical cues. The results show that reproductive chemical communication is in operation in both test species. The cross species interaction indicates that pheromone pollution may represent an additional impact of non - native species introductions.

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CHAPTER 1. INTRODUCTION

1.1. CHEMICAL COMMUNICATION

Chemical signals are used by a diverse range of taxa. This includes mammals, (Brennan & Keverne, 2004) fishes, (Brönmark & Hansson, 2000) reptiles, (LeMaster & Mason, 2002) invertebrates (Stebbing *et al.,* 2003) and amphibians (Kikuyama *et al.*, 2002). These chemical signals facilitate a number of key behaviours such as predator avoidance, (Friesen & Chivers, 2006) migration, (Sorensen *et al.*, 2005) shoaling, (Mann *et al.*, 2003) foraging (Wyatt, 2003) and reproduction (Kobayashi *et al.*, 2002). Even bacteria such as *Enterococcus faecalis* (Schleifer & Kilpper-Balz) are known to produce an agent that promotes aggregation behaviour that could represent a sex pheromone (Dunny *et al.*, 1978). Due to the importance of chemical signals in animal behaviour, it is surprising that the first pheromone was only discovered in the last 50 years (Butenandt *et al.*, 1959; Karlson & Luscher, 1959).

Pheromones are defined here as 'an odour or mixture of odours released by the sender that evokes in the receiver(s) adaptive, specific, and species–typical response(s), the expression of which need not require prior learning or previous experience' (Sorensen & Stacey, 2004). This definition accounts for new research (using teleost fish) on pheromone interactions between individuals. Notably it is now known that pheromones can exist as a number of different compounds in vertebrates as well as in insects (Poling *et al.*, 2001) and that specialisation of the odour(s) to play a role in chemoreception (i.e. the use of specially designed compounds) is not required (Stacey & Sorensen, 2002). For example, most reproductive pheromones are derived from hormones (see section 1.3).

1.2. SEX PHEROMONES

Sex pheromones are involved in mating behaviour and mate selection and have been identified in insects for some time (Butenandt *et al.*, 1959). Recent research has indicated that in certain species such as the firefly *Phosphaenus hemipterus* (Fourcroy) sexual communication is exclusively based on chemical signalling (de Cock & Matthysen, 2005). Although presently more is known about insect olfactory systems than that of vertebrates and therefore forms the bulk of olfactory understanding, vertebrate chemical communication has now become a focus of research. In urodeles, pheromone communication has been demonstrated through products of selected glands that undergo increased development during the reproductive period. A female attracting pheromone called sodefrin has been identified in the male red–bellied newt *Cynops pyrrhogaster* (Boie) (Kikuyama *et al.*, 1995). Similarly a sodefrin like chemical was found to attract female sword-tailed newts *Cynops ensicauda* (Hallowell) (Yamamoto *et al.*, 2000). In anurans, chemical communication has also been demonstrated, with a sex pheromone identified in the magnificent tree frog *Litoria splendida* (Tyler, Davies & Martin) (Wabnitz *et al.*, 1999).

The active compounds and their metabolites used as sex pheromones are in some instances common for a number of taxa. Asian elephants use the same compound to signal readiness to mate, as that used by over 100 species of butterfly and moth (Rasmussen *et al.*, 1996). This suggests that the compounds used as sex pheromones may be commonly conserved through different taxa. Due to this, the study of olfactory systems in early vertebrates, such as fish, is relevant to the entire animal kingdom. This is particularly relevant in vertebrates where the components of the olfactory system (anatomical, cellular and biochemical) have remained highly conserved through evolution (Stacey *et al.*, 2003).

In addition to being a blueprint for sex pheromone systems in vertebrates, studies regarding reproductive chemical communication in fishes have other merits. In threatened

species where natural mating behaviour is rare, or in commercial farms where mating behaviour is induced, understanding more about the processes underlying pheromone mediated reproductive behaviour could be crucial. Basic knowledge could also be used for the control of invasive species where pheromone traps could be a new tool available to ecosystem management. This chapter will specifically review the range of behaviours mediated by sex pheromones, the species specificity of pheromones, the compounds used as reproductive pheromones and the potential of sex pheromones in conservation and is based on a published review by Burnard *et al.*, (2008).

1.3. SEX PHEROMONES IN FISHES

The nature of the aquatic environment lends itself well to chemical communication. Here, poor visibility (due, for example, to turbidity or the presence of weed patches) can render visual signals obsolete, but water can mediate the external transfer of information. Chemical signals facilitate a range of behaviours in fish including predator avoidance (alarm) (Brown *et al.*, 1995; Brown *et al.*, 2001), social recognition (Moore *et al.*, 1994; Olsen, 1999), shoaling (Ward *et al.*, 2004; Webster *et al.*, 2008) and migration (Sorensen & Vrieze, 2003; Baker *et al.*, 2006). However most described fish pheromones to date are associated with reproductive activity (Table 1.1.).

Table 1.1. Known sex pheromones in fishes

Compound	Species	Effect	Reference
Prostaglandins			
Prostaglandin F2α	Atlantic salmon (*Salmo salar*)	Male priming	Moore & Waring (1996)
	Arctic charr (*Salvelinus alpinus*)	Attracts females and elicits spawning behaviour	Sveinsson & Hara (1995)
	Brown trout (*Salmo trutta*)	Female pre-spawning behaviour	Laberge & Hara (2003)
	Goldfish (*Carassius auratus*)	Elicits male sexual behaviour	Kobayashi *et al.*, (2002)
	Lake whitefish (*Coregonus clupeaformis*)	Increases locomotor activity in males and females	Laberge & Hara (2003)
13,14-dihydro-15-keto-	Cobitid loach (*Misgurnus*	Elicits male sexual behaviour	Ogata et al., (1994)
prostaglandin F2α (F2α	*anguillicaudatus*)	Increases locomotor activity in males and females	Laberge & Hara (2003)
metabolite)	Brown trout (*Salmo trutta*)		
15-keto-prostaglandin	Goldfish (*Carassius auratus*)	Elicits male sexual behaviour	Kobayashi *et al.*, (2002)
F2α	Lake whitefish (*Coregonus clupeaformis*)	Increases locomotor activity in males and females	Laberge & Hara (2003)
(F2α metabolite)	Atlantic salmon (*Salmo salar*)	Male priming	Moore & Waring (1996)

Steroids un-conjugated			
Etiocholanolone	Round goby (*Neogobius melanostomus*)	Increases ventilation rate in males and females	Murphy *et al.*, (2001)
		Female attractant (possible)	Arbuckle *et al.*, (2004)
11-Ketotestosterone	Round goby (*Neogobius melanostomus*)	Female attractant (possible)	Arbuckle *et al.*, (2004)
17,20β-P	Rainbow trout (*Oncorhynchus mykiss*)	Unknown effect in male and female	Vermeirssen & Scott (1996)
	Goldfish (*Carassius auratus*)	Elicits male sexual behaviour and physiological priming	Kobayashi *et al.*, (2002)
	Roach (*Rutilus rutilus*)	Unknown effect in males and females	Lower *et al.*, (2004)
Testosterone	Three spot gourami (*Trichogaster trichopterus*)	Unknown male effect	Becker *et al.*, (1992)
	Yellowfin baikal sculpin (*Cottocomephorus grewingki*)	Elicits female spawning behaviour	Katsel *et al.*, (1992)
Estrone	Round goby (*Neogobius melanostomus*)	Increases ventilation rate in male	Murphy *et al.*, (2001)
17β-estradiol	Round goby (*Neogobius melanostomus*)	Increases ventilation rate in male	Murphy *et al.*, (2001)

Steroids conjugated			
Etiocholanolone (sulphated)	Round goby (*Neogobius melanostomus*)	Female attractant (Possible)	Arbuckle *et al.*, (2005)
Etiocholanolone (glucuronidated)	African catfish (*Clarias gariepinus*)	Female attractant (Possible)	Resink *et al.*, (1989)
	Black goby (*Gobius jozo*)	Female attractant	Colombo *et al.*, (1980)
17,20β-P (sulphated)	Goldfish (*Carassius auratus*)	Elicits male sexual behaviour	Kobayashi *et al.*, (2002)
	Rainbow trout (*Onchorhynchus mykiss*)	Unknown effect in males and females	Vermeirssen & Scott (1996)
	Hill trout (*Barilius bendelisis)*	Male priming pheromone	Bhatt & Sajwan (2001)
17,20β-P (glucuronidated)	Rainbow trout (*Onchorhynchus mykiss*)	Unknown effect in male and female	Vermeirssen & Scott (1996)
	Roach (*Rutilus rutilus*)	Unknown effect in males and females	Lower *et al.*, (2004)
	Zebrafish (*Brachydanio rerio*)	Induces ovulation in females	Van Den Hurk & Resink (1992)
Dehydroepiandrosterone (glucuronidated)	Round goby (*Neogobius melanostomus*)	Increases ventilation rate in males and females	Murphy *et al.*, (2001)
Other			

7α-12α,24-trihydroxy-5α-cholan-3-one 24 sulphate (bile acid)	Sea Lamprey (*Petromyzon marinus*)	Female sexual attractant	Li *et al.*, (2002)

1.4. FISH REPRODUCTIVE PHEROMONE SYSTEMS

The olfactory system of fish can be divided into three main components. A peripheral part which is the site of the olfactory organ and houses the olfactory bulb and olfactory epithelium. An intermediate part comprising of an anterior olfactory nucleus and a central part located largely in the paleocortical region of the brain (Zeiske *et al.*, 1992). The central component is at present poorly studied and can not be discussed without an extensive knowledge of neurobiology, outside the scope of this review (see Sorensen & Caprio, 1997). The peripheral olfactory system however has been the subject of much research.

The basic model of a single olfactory peripheral organ consists of an anterior naris, an olfactory chamber containing Olfactory Sensory Neurons (OSNs) and a posterior naris (Belanger *et al.*, 2003). Each naris is separated by a nasal bridge. In teleosts this structure is paired (two organs) and located on the snout of the fish. Water flows one way through the olfactory chamber (anterior to posterior naris) generated by an extension(s) called the nasal or ventilation sac (Belanger *et al.*, 2003) and or associated cilia. Located on the floor of the olfactory chamber is a convoluted structure called the olfactory rosette. This comprises of individual lamella containing OSNs and is covered by the olfactory epithelium.

The size and shape of the olfactory rosette, and the number of lamella are variable in different species, creating a wide range of viable forms (Zeiske *et al.*, 1992). Soluble odourants flow over the surface of the OSNs where the molecules interact with G-protein-regulated olfactory receptor cells. The large surface area provided by the olfactory lamellae allows many OSNs to be situated in the olfactory chamber. OSNs project from the olfactory epithelium into the olfactory bulbs, where the axons of common receptor types terminate and synapse with mitral cells (Yoshihara *et al.*, 2001) in aggregations termed glomeruli. From the bulbs, mitral cells project *via* the medial and lateral olfactory tracts and terminate in specific regions of the telencephalon and hypothalamus (Zeiske *et al.*, 1992).

The original unit of a species reproductive function is its Hypothalamo-Pituitary-Gonadal axis (HPG). This is capable of internal regulation and response to external stimuli. Since hormonal products (and their derivatives) are exogenous stimuli, a feedback loop incorporating auto stimulation and indirect stimulation via conspecific pheromone release is required (Stacey & Cardwell, 1995). This latest version of the model requires the acknowledgement of a hormonal system incorporating conspecifics linked via water borne external hormones, distinct from viewing the system as involving only one individual.

It has been suggested (Sorensen & Scott, 1994) that hormonally-derived fish pheromones have evolved along similar processes to that proposed by Kittredge *et al.*, (1971) for marine invertebrates. Here, this type of pheromone evolved due to pre-adaptation of hormones to function as pheromones. Several themes promote this, including the normal production and release of these hormones, the importance of detecting them (due to the information contained on physiological state) and the ease in detecting them. This latter point is based on the hypothesis that only a single point mutation could be required to express hormone receptors externally on chemosensory receptor cells (Sorensen & Scott, 1994).

Three stages characterise the evolution of chemoreception using this hypothesis. Stage one is the excretion of hormones that have no pheromone function. This occurs due to the normal release of these hormones to regulate internal systems. Stage two constitutes spying, characterised by chance expression of hormone receptors on olfactory tissue. This is the detection of hormones released by conspecifics that allows the transfer of information to the receiver. Stage three is the evolution of *bona fida* communication. This is the permitted release of pheromones to benefit both donor and receiver (Liley, 1982). Evidence suggests that different fish species are at different stages of this evolution process (Sorensen & Scott, 1994). The homologous and evolutionary conserved origin of fish reproductive endocrine systems provides a great opportunity to model how chemoreception has evolved and diverged

over time in different species with distinct mating strategies. This is why fish are particularly good taxa for the study of chemoreception.

1.4.1. What information can sex pheromones provide?

1) *Sex discrimination* - The most basic requirement for an individual searching for a mate is to identify one of the correct sex. Pheromones produced by both females and males allow conspecifics to be distinguished on the basis of sex (see Liley, 1982).

2) *Mating* - Even when a conspecific of the required sex has been attracted, pheromones can also be used to allow identification of a partner with the physiological state of readiness necessary for reproduction to proceed. For example, 11-ketotestosterone, a steroidal androgen that controls reproductive/behavioural cycle is produced by male three-spined stickleback *Gasterosteus aculeatus* L. Females can directly detect this compound which signals readiness to mate (Häberli & Aeschlimann, 2004).

3) *Health* - The major histocompatibility complex has been shown to affect mate choice decisions (Milinski, 2003). It is possible that females avoid close relatives, choosing instead males with non-matching, complimentary immune genes that impart strong defence against parasites and disease. Fish are rapidly becoming the new model organism for study in this field.

1.4.2. Compounds used as sex pheromones

Most identified reproductive pheromones are derived from naturally released hormones or bile acids. The former consists of either free or conjugated C18, C19 and C21 steroids, prostaglandins or their metabolites. The water solubility of such compounds varies and so certain pheromone types may be more likely, in evolutionary terms, to function as sex pheromones in selected species (Liley, 1982). As research continues, different sex

pheromones are being identified that do not derive from these hormonal groups. Spermiating male sea lamprey *Petromyzon marinus* L. for example, release a novel bile acid 7α, 12α, 24-trihydroxy-5 α-cholan–3–one 24-sulphate (Li *et al.*, 2002). This acts as a long distance female attractant. In the puffer fish *Fugu niphobles* (Jordan & Snyder) tetrodotoxin has also been shown to function as a mate attracting sex pheromone, but unlike the sea lamprey it is released by females (Matsumura, 1995).

Although not ideal for classification, pheromones can be divided into releaser and primer types (Wilson & Bossert, 1963). Releaser pheromones induce rapid behavioural changes in the recipient, like increased activity. Primer pheromones induce more long term physiological changes, such as milt production (Moore & Waring, 1996). Even though different species can produce and release the same pheromone, their effects may vary. For example, conjugated (sulphated) 17α,20β-dihydroxy-4-pregnen-3-one functions as a releaser in male goldfish *Carassius auratus* L. (Kobayashi *et al.*, 2002) and a primer in hill trout *Barilius bendelisis* (Hamilton) (Bhatt & Sajwan, 2001). Secondly, a pheromone is not restricted to a specific sex. In the Arctic charr *Salvelinus alpinus* L., it is the males that release prostaglandin F2α (PGF) to elicit a response in females (Sveinsson & Hara, 1995), whilst in Atlantic salmon, females release PGF to prime males (Moore & Waring, 1996). Due to these difficulties in classifying a pheromone as a primer or releaser type, Stacey and Sorensen, (2006) suggest that this classification be only applied to the effects they evoke. Hence, a specific compound could evoke primer effects in one species and releaser effects in another.

1.5. SPECIES SPECIFICITY

Generally it is accepted that closely related fish species have similar pheromone systems (compounds and effects) and distantly related species have dissimilar ones. This seems to be the case at least in hormonally-derived sex pheromones (Irvine & Sorensen,

1993). The common carp *Cyprinus carpio* L. and goldfish *Carassius auratus* L. appear to share common steroidal pheromone systems. Sensitivity to17α, 20β-dihydroxy-4-pregnen-3v-one, 17α,20βP–sulphate and androstenedione occurs in both species (Irvine & Sorensen, 1993). Similar results are shown regarding sensitivity to 17α, 20β-dihydroxy-4-pregnen-3-one and prostaglandin $F_{2\alpha}$ in both the goldfish and crucian carp *Carassius carassius* L. (Bjerselius & Olsen, 1993). As closely related species share sex pheromones, hybridisation between them may also occur. In mate choice, pheromones are used to attract or distinguish between potential conspecific mates. However if two species have very similar pheromones, heterospecific mates may be attracted by accident, or alternatively the pheromone of one species may confuse another and prevent the accurate identification of a suitable conspecific. Either way hybridisation may be the outcome. For example, in the goldfish and the crucian carp, interbreeding does occur (Hänfling *et al.*, 2005). In both brook trout *Salvelinus fontinalis* (Mitchill) and brown trout *Salmo trutta* L. equal sensitivity to prostaglandin $F_{2\alpha}$ and its derivatives (Essington & Sorensen, 1996) suggests similar sex pheromone systems, and hybridisation is common.

Given the limited number of compounds used as sex pheromones, hybridisation between closely related species that share sex pheromones could be expected to be more common than observed. However, it is likely that there are a variety of precursors to reproduction and the event is not subject to one underlying factor. Other signals may play a significant role in orchestrating spawning, including visual, auditory, tactile and electrical cues (Irvine & Sorensen, 1993; Olsén *et al.*, 2000). Differences in the timing of the mating season could also be a determinant. There may be no overlap in spawning periods or diurnal variations when spawning occurs, preventing interaction between reproductively active heterospecifics.

It could be expected that closely related allopatric species share common sex pheromones and sympatric species do not (Irvine & Sorensen, 1993; Essington & Sorensen, 1996). This is true for certain species such as the Common carp and the goldfish (allopatric). However, for the masu salmon *Oncorhynchus masou* (Gunther) and rainbow trout *Oncorhynchus mykiss* (Walbaum), which are also allopatric species, each releases species-specific sex pheromones (Yambe & Yamazaki, 2001). Geographical isolation of two closely related species therefore does not necessarily mean both will share a common sex pheromone(s). Likewise, sympatric species do not always exhibit different sex pheromones. Males of both Atlantic salmon *Salmo salar* L. and brown trout show physiological response to ovarian fluid and urine of conspecific and heterospecific females (Olsén *et al.*, 2000).

In insects, specific blends of compounds are often used. The female tobacco hawkmoth *Manduca sexta* L. produces a mixture of 12 compounds which are all 16 or 18 carbon aliphatic aldehydes (Tumlinson *et al.*, 1989). Only some of these compounds are necessary for male attraction, and a component is used to reduce cross–species attraction with closely related forms (Christensen *et al.*, 1994). Authors such as Poling *et* al., (2001) have noted that male goldfish can discriminate between the different components of the female pheromone. It is possible that fish may discriminate between specific mixtures of compounds that may vary slightly between closely related species. If species can discriminate between signals produced by conspecifics and that of heterospecifics, it is likely to be by this approach (Sorensen & Scott, 1994). This is particularly valid for the use of prostaglandins where its almost 'universal' action would mean that an individual would not be able to discriminate between conspecifics and heterospecifics (Stacey & Cardwell, 1995).

1.6. THE USE OF SEX PHEROMONES IN CONSERVATION

The biodiversity of aquatic ecosystems is vulnerable to the introduction of non-native species (Sala *et al.*, 2000), posing a threat through predation of native species, resource competition, introduction of new diseases (Gozlan *et al.*, 2005), or alteration of the environment (Manchester & Bullock, 2000). Any technique that could help mitigate this problem is a potentially important tool in conservation. The sea lamprey control programme (Great Lakes) has enjoyed success by the application of an integrated pest management (IPM) approach. Here different life stages are targeted simultaneously by a variety of methods including the use of toxins and the introduction of sterile males.

The high fecundity of sea lamprey and the damaging impact on native fishes has led to the search for more methods of control. Petromyzonol sulphate (Li *et al.*, 1995) a compound that induces homing behaviour has already been highlighted for potential use. This could be used in a number of ways, including diverting migratory adults to streams where they are unlikely to reproduce (Sorensen *et al.*, 2003). Application of the recently discovered 7α, 12α, 24-trihydroxy-5α-cholan-3-one 24-sulphate may prove successful. This is a potent pheromone that induces searching behaviour in ovulated females. As it is a bile acid and more water soluble than steroids, it can be detected from a greater range (up to 65 m, Li *et al.*, 2002). This makes it an ideal candidate for use in IPM, and could be used to trap mature females. Application could result in a shift in the sex ratio of the species causing severe competition for mates (Corkum, 2004).

Pheromones play a vital role in the reproduction of another invasive species, the round goby *Neogobius melanostomus* (Pallas). Originating from the Black and Caspian Seas this fish has already been reported in the Mississippi River basin (Jude *et al.*, 1992) and has spread to the Great Lakes. This could lead to a loss of biodiversity due to competition for resources in species such as mottled sculpin *Cottus bairdi* (Girard) (Dubs & Corkum, 1996)

and egg predation in lake trout *Salvelinus namaycush* (Walbaum) (Chotkowski & Marsden, 1999) and lake sturgeon *Acipenser fulvescens* (Rafinesque) (Nichols *et al.*, 2003). In the laboratory reproductive females are attracted to odours released by mature males (Corkum *et al.*, 2006). Electro-olfactory gram (EOG) analysis on over 100 synthetic steroids and prostaglandins found that 19 steroids elicited a response (Murphy *et al.*, 2001). The potential for pheromone use in the control of this species therefore appears viable.

There is encouragement for the use of sex pheromones in the control of non-native vertebrate aquatic species. Positive results were achieved in trials of traps baited with the water conditioned by reproductive females of the signal crayfish *Pacifastacus leniusculus* (Dana) (Stebbing *et al.*, 2004). These trials are based on previous research where water conditioned by reproductive females induced courtship and mating behaviour in males (Stebbing *et al.*, 2003). However, currently there is no understanding of the structural identity of the pheromone and this could hinder mass application for pest control.

Reproductive pheromone application could also have a potential use in breeding programmes. This is particularly relevant for species that to date have poor reproductive success in captivity. If breeding could be induced in tropical fish for example, the level of wild stock caught could be reduced. Commercial fish farms and hatcheries could also profit from their use. This could eventually enable managers to design a breeding programme that suits them, rather than being based exclusively around the fish normal reproductive cycle. For such pheromone application to be successful, identification of the pheromones used in each species is required.

1.7. DISCUSSION

Sex pheromone systems occur in a large number and diverse range of fish species (see Burnard *et al.*, 2008). Although research in this field has progressed there is a lot of

scope for further study. It is not known if the signalling mechanism used in the best understood fish species, the goldfish, is a common mechanism for pheromone signalling in fish. Greater understanding of sex pheromone activity can only be achieved through detailed study of other fish species. It may be that deep-sea fishes that are deprived of visual senses rely heavily on sex pheromone signalling. Sharks and rays with their sharp olfactory senses may use chemoreception as a major means of communication.

Due to the potential impact to ecosystems caused by non-native species, research on the reproductive pheromones that they use could be an important tool for non-native species management. Investigations should include characterising the olfactory response to stimuli in selected invasive species and practical methods (application) in their control. Pheromone interaction between species (see chapter 5) should also be investigated as invasive species could indirectly use this as an aid to become established in an ecosystem. For example, it is possible that the sex pheromones used by one species could interfere with chemical communication in another species. Specificity between pheromone systems may not have developed due to previous geographical isolation. As sex pheromones in freshwater fish (in investigations so far) are derived from hormones or bile acids (which are conserved through different taxa), it is possible that species use the same or similar compounds as sex pheromones.

Studies of sex pheromones can incorporate the use of the Electro-Olfactogram (EOG) technique (see chapter 7). EOG allows the individual compounds that elicit an electrophysiological response to be identified and isolated from mixtures of compounds when they are analytically separated. EOG technique also enables a wide range of chemicals to be tested for an active response by fish. Similarly, compounds isolated from one species can be tested for their effect on another. The effects of pollution on pheromone activity can also be studied using this approach. As EOG provides validation of behavioural experiments

(chemical signals that elicit a behavioural responses will also elicit an electrophysiological response) further study should incorporate EOG in combination with other techniques such as high performance liquid chromatography (HPLC) and behavioural assays (see chapters 3, 5, 6 and 7) to accurately map olfactory systems.

Sex pheromone systems occur throughout the animal kingdom. Continuing study using fish has particular merits. Some species are a good biological model to study under laboratory conditions and generally fish have limited behaviour patterns compared to other vertebrates. As sex pheromones have been identified in primitive fishes such as Elopiformes, it is expected that they are common amongst all species of fish (Stacey *et al.*, 2003) so the scope for further study in this field is vast. It is clear that new advances in the field of fish behaviour and fish ecology will be heavily influenced by the knowledge gained from fish pheromones.

1.8. MODEL SPECIES: TOPMOUTH GUDGEON (*PSEUDORASBORA PARVA*)

The topmouth gudgeon *Pseudorasbora parva* (Temminck & Schlegel) is a benthic cyprinid native to Japan, China and Korea. They were accidentally introduced into Romanian ponds in 1960 alongside Chinese carp species imported for aquaculture and subsequently spread rapidly throughout Europe via the Danube and Rhine watercourses (Weber, 1984). Further introductions (accidental and deliberate) in France (Allardi & Chancerel, 1988) and England (Domaniewski & Wheeler, 1996) have resulted in topmouth gudgeon being found across continental Europe less than 40 years after being initially recorded (Gozlan *et al.*, 2002). In addition to being recognised as a highly invasive fish species in Europe, topmouth gudgeon has been identified in Turkey, (Wildekamp *et al.*, 1997) Kazakhstan, (Arnold, 1990) Uzbekistan, (Arnold, 1990) and Algeria (Perdices & Doadrio, 1992). Worldwide, a

combination of social, economic and biological factors has fuelled their invasion with 32 countries invaded in less than 50 years (Gozlan *et al.*, 2002). They are now regarded as a highly invasive pest species across the globe (Gozlan *et al.*, 2002).

Various threats to native fish have been highlighted due to the introduction of topmouth gudgeon. They are healthy carriers of the rosette agent which has been shown to cause mass mortality to the European cyprinid sunbleak *Leucaspius delineatus* (Heckel) under experimental conditions (Gozlan *et al.*, 2005). They are also vectors for the parasites *Anguillicola crassus* (Cesco *et al.*, 2001) and *Clinostomum complanatum* (Aohagi *et al.*, 1992) in addition to being a carrier of pike fry rhabdovirus (Ahne & Thomsen, 1986). Topmouth gudgeon occupy the bentho-pelagic zone and have a broad diet consisting of zooplankton, algae, invertebrates, molluscs and also the eggs and larvae of other fishes (Xie *et al.*, 2000). In the UK, dietary overlap with juvenile native species (Beyer, 2008) could result in competition for food resources. Topmouth gudgeon are included on the Import of Live Fish Act (ILFA) list (Defra, 1998) making it an offence to move the species without a licence.

Topmouth gudgeon have evolved a life-history strategy, distinct from most other cyprinids, that aids successful colonisation of water bodies (Yan & Chen, 2009). They are sexually mature at one year old and females lay several batches of eggs throughout the spawning season (April to July in the UK but differs throughout its geographical range depending on climate) which are guarded by males until they hatch (Dussling & Berg. 2001; Rosecchi *et al.*, 2001). Larvae survival rates are enhanced as the time period over which the broods are produced is spread across an extended period decreasing susceptibility due to changes in environmental conditions (Gozlan *et al.,* 2003b). These factors combined with a limited life span (less than five years) ensure a high population turnover that promotes colonisation and establishment (Katano & Maekawa, 1997; Pinder *et al.*, 2005; Yan & Chen, 2009).

The species exhibit sexual dimorphism with the male being larger than the female (Okado, 1961; Britton *et al.*, 2007; 2008) (Fig. 1.1). During the spawning season males exhibit secondary sexual characteristics including tubercules around the mouth (Nichols, 1929) (Fig. 1.2) and a dark body colouration. At this time males establish and guard primitive nests. Topmouth gudgeon show considerable variability in their choice of spawning substrate which includes the underside of rocks and floating macrophytes (Maekawa *et al.*, 1996). Topmouth gudgeon exhibit a hierarchical dominance mating strategy: bigger males guard and defend larger territories (Maekawa *et al.*, 1996). Female mate choice is determined by the size of the male, and favours males with a large body size (Maekawa *et al.*, 1996). When a nest site has been established, males leave in search of a gravid female. Male courtship behaviour is not well documented but is known to include approach and leading behaviour and a zig zag swimming motion performed in close proximity to females (Maekawa *et al.*, 1996). Females then attach their eggs to the substrate, followed by the male which then releases sperm. Females spawn with several different males in one day and deposit several batches of eggs in each nest (Maekawa *et al.*, 1996).

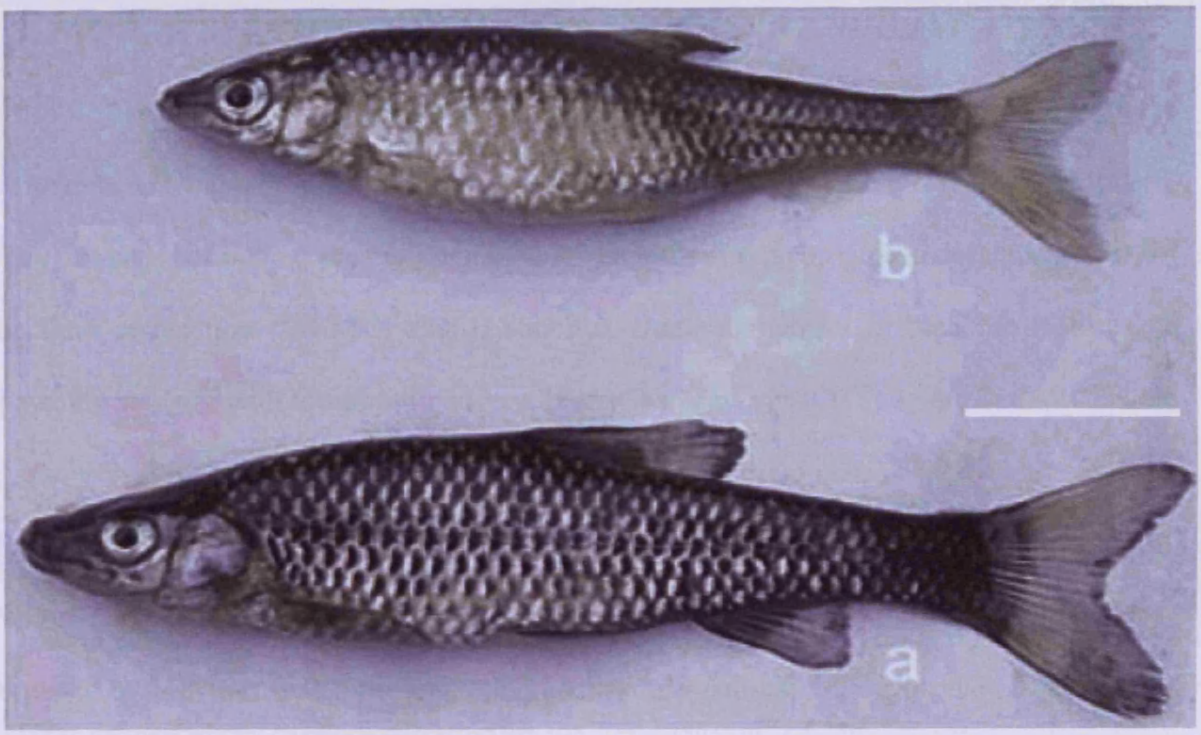

Figure 1.1 Topmouth gudgeon *Pseudorasbora parva* male (a) and female (b) showing differences in morphology such as body colouration and female abdomen dilation. The white bar is 10 mm long. Photo by R. E. Gozlan.

Figure 1.2. Male topmouth gudgeon *Pseudorasbora parva* with white tubercules around the mouth (a secondary sex characteristic). Photo by author.

1.9. MODEL SPECIES: SUNBLEAK (*LEUCASPIUS DELINEATUS*)

Sunbleak originate from continental Europe and Russia and are distributed from the Caspian Sea to the North Sea and from the Volga to Brittany, France (Gozlan *et al.*, 2003b). They are now considered area and vulnerable across their native range under appendix III of the Bern convention (WCMC, 1996). Sunbleak share similarities in both life history and spawning strategy with topmouth gudgeon (Farr-Cox *et al.*, 1996). They are sexually mature at one year, grow to a maximum of 8 cm and exhibit paternal care of their eggs. However topmouth gudgeon males have a spawning strategy based on male dominance and sunbleak exhibit allopaternal care (Gozlan *et al.*, 2003a). In sunbleak, nest sites containing eggs fertilised by previous males are often adopted by new suitors, who evict the original males from the nest. Males guard territories around the leaves and stems of aquatic macrophytes such as water lilies. Gravid females deposit strips of eggs in the nest which are subsequently guarded by the male. During this time males encourage other females to deposit their eggs in the same nest which are subsequently fertilised (Gozlan *et al.*, 2003a). Females are larger than males and during spawning, reproductive females can be identified by their swollen ovipositors (Fig. 1.3).

Sunbleak are gregarious and shoal at the top of the water column where they feed on zooplankton and terrestrial insects. They prefer still waters and slow flowing rivers but can use fast waters as a means of dispersal (Gozlan *et al.*, 2003b). They originate from continental Europe and are considered rare and vulnerable in most of their native range (Lelek, 1987). In the UK, since their escape from an ornamental fish farm in Hampshire (Farr-Cox *et al.*, 1996), sunbleak have spread throughout the Somerset levels and areas of Dorset (Farr-Cox *et al.*, 1996). Sunbleak are included on the Import of Live Fish Act (ILFA) list (Defra, 1998) making it an offence to move the species without a licence.

In the UK, threats to native fish due to the introduction of sunbleak are not well documented. In this species, energy reserves that are available in their first year for growth are reallocated to gonad production in subsequent years (Gozlan *et al.*, 2003b). Consequently this slow growth results in combined age classes competing with juvenile native species for food resources (Gozlan *et al.*, 2003b). There is however no evidence of piscivory in sunbleak (Gozlan *et al.*, 2003b). In parallel with topmouth gudgeon, the fast reproductive rate of sunbleak could result in the species becoming numerically dominant over native species within a short period of introduction (Gozlan *et al.*, 2003b).

Although reproduction has been preliminary documented in topmouth gudgeon (Maekawa *et al.*, 1996) and sunbleak (Gozlan *et al.*, 2003a), the role of chemical communication in facilitating this event is unknown. Due to the importance of sex pheromones in other cyprinids, notably the goldfish, (see Kobayashi *et al.*, 2002) and the complexity of reproductive behaviour in both topmouth gudgeon and sunbleak, it could be expected that chemical signals play a major role during spawning. As both species are invasive in some part of their non-native range and there is potential for population size to be controlled using sex pheromones, research in this area is of particular importance.

Figure 1.3. Female sunbleak *Leucaspius delineatus* with swollen ovipositor (indicated by the arrow). The white bar is 10 mm long. Photo by A.C. Pinder.

1.10. INTERACTION BETWEEN TOPMOUTH GUDGEON *PSEUDORASBORA PARVA* AND SUNBLEAK *LEUCASPIUS DELINEATUS*

Sunbleak has experienced high declines in their native range over the past 40 years (Gozlan *et al.*, 2009) and are currently listed on the IUCN red list of vulnerable species (WCMC 1996). The observed decline in sunbleak populations coincided with the spread of topmouth gudgeon through continental Europe and Gozlan *et al.*, (2005) hypothesised that topmouth gudgeon had contributed to sunbleak's decline. In order to investigate the interaction between these two species, Gozlan *et al.*, (2005) cohabited sunbleak and topmouth gudgeon in controlled indoor cohabitations. Cohabitation of sunbleak with topmouth gudgeon led to spawning inhibition, emaciation and high mortality (69 %) in sunbleak. Examination of moribund fish revealed the presence of the rosette-like-agent parasite which was associated with 67 % of sunbleak mortalities. The rosette-like-agent was later identified as *Sphaerothecum destruens* (Gozlan *et al.* (2009) which is an intracellular parasite that has been reported in Chinook

salmon *Oncorhynchus tshawytscha* (Walbaum). Infection of Chinook salmon with *S. destruens* did not inhibit spawning (Arkush *et al.*, 1998). Therefore, the spawning inhibition observed in sunbleak may not be due to the presence of *S. destruens* and the possibility that the observed spawning inhibition was the result of a sex pheromone interaction between the two species could not be excluded.

1.11. AIMS AND OBJECTIVES

The aim of this thesis is to investigate reproductive chemical communication in the cyprinids topmouth gudgeon and sunbleak. The hypotheses tested are:

1) Topmouth gudgeon and sunbleak use reproductive chemical communication.

Topmouth gudgeon and sunbleak males guard nests and then search for receptive females therefore it is hypothesised that reproductive males release chemical cues to elicit behavioural responses in reproductive females. Chapter 3 will address the possible function of a sex pheromone in topmouth gudgeon. In addition, swimming activity and swimming vagility (tendency to move/the degree to which a donor fish moves in its environment) will be quantified in response to conditioned water in topmouth gudgeon and sunbleak to determine recognition of reproductive chemical cues (chapter 4).

2) Reproductive chemical cues released by topmouth gudgeon.elicit behavioural responses in reproductive sunbleak.

The possible effect that a sex pheromone released by one species has on another species is important due to the translocation and establishment of non-native fish. Chapter 4 will test the responses of sunbleak to cues released by topmouth gudgeon and the responses of topmouth gudgeon to cues derived from sunbleak. A bioassay quantifying swimming activity

and swimming vagility will determine if interspecific recognition of reproductive chemical cues occur between these species (chapter 5).

3) Solid Phase Extraction (SPE) elute and High Performance Liquid Chromatography (HPLC) separated fractions derived from reproductive male topmouth gudgeon elicit behavioural responses in reproductive females.

Many studies to date have successfully isolated sex pheromones from conditioned water (Li *et al.,* 2002; Belanger *et al.,* 2004; Sorensen *et al.,* 2005a). Chapter 6 describes a bioassay testing whether active compounds can be isolated in topmouth gudgeon. On identification of a response, elute separated by retention time using HPLC will be tested to determine active fractions (chapter 6).

4) Conditioned water derived from reproductive male topmouth gudgeon elicits Electro-Olfactory Gram (EOG) responses in reproductive females.

The EOG technique coupled with behavioural studies provides conclusive evidence of pheromonal recognition by receivers. Chapter 7 will elucidate whether water conditioned by donors are recognised by both sexes in topmouth gudgeon.

CHAPTER 2. GENERAL MATERIALS AND METHODS

This chapter describes animal collection, gender identification and the general experimental techniques used throughout the thesis.

2.1. ANIMAL COLLECTION AND IDENTIFICATION

2.1.1. Collection of topmouth gudgeon *Pseudorasbora parva*

In May 2006 - 2009 topmouth gudgeon individuals (approximately 1000 in total) were obtained from the Environment Agency (EA). Sampled sites were Elm Hag Lake (Yorkshire, England: 54°12'37" N; 1°10'41" W and Larton Livery (Cheshire, England: 53°22'31" N; 3°08'22" W). Fish were caught by seine netting and transported by commercial carrier to the Centre for Ecology and Hydrology (CEH) tank facilities (see section 2.3).

2.1.2. Identification of gender and reproductive status

Sexual dimorphism in the species allows accurate identification of gender (see chapter 1, section 1.8). Dominant reproductive males guarded a primitive nest (i.e. ceramic tile) and were identified by their dark coloration, and tubercules (see Fig. 1.2). Non reproductive males did not perform guarding behaviour and did not have secondary sex characteristics. Reproductive females were distinguished from non reproductive females by their swollen abdomen and yellow colouration. Gender was confirmed on completion of trials by dissection and gonad identification. Fish were killed by Schedule 1 methods as per the 'Animals (Scientific Procedures) Act 1986' with an overdose of 2-Phenoxyethanol (2 PE) followed by severance of the spinal cord at the base of the skull (Home Office, 1986a; b). Females were distinguished from males by the presence of eggs in their ovaries (all females

regardless of reproductive status possess eggs in their ovaries). Gonadal Somatic Index (GSI) is used in fish biology to distinguish between reproductive and non reproductive fishes (see Cole & Smith, 1987; Frade *et al.*, 2002; Belanger *et al.*, 2004). GSI is the ratio of gonad weight to body weight and is used to estimate reproductive condition. GSI was calculated for each fish according to:

$$GSI = 100(Wg/(Wt-Wg))$$

where Wg is the gonadal weight and Wt is the total weight of the fish.

2.1.3. Collection of sunbleak *Leucaspius delineatus*

In April 2006 and 2008 approximately 1000 sunbleak in total were collected using seine netting from Stoneham lakes (Eastleigh, England: 50°57'14" N; 1°22'56" W). Fish were collected by the CEH fish ecology group and then transported to CEH tank facilities (see section 2.3 for description of holding facilities). During the summer of 2007 and 2009 it was not possible to obtain stock due to low population numbers of sunbleak in the UK. All fish used in experiments did not exhibit any external signs of rosette agent infection. Infected fish emaciate and typically die within two weeks of exposure to the agent (Gozlan *et al.*, 2005).

2.1.4. Identification of gender and reproductive status

Sexual dimorphism in the species allows accurate identification of gender (see chapter 1 section 1.9). Dominant reproductive males guarded a primitive nest (i.e. artificial lily). Non reproductive males did not perform guarding behaviour. Reproductive females were distinguished from non reproductive females by their swollen ovipositors (Fig. 1.3, chapter 1).

Gender was confirmed on completion of trials by dissection and gonad identification. Fish were killed by Schedule 1 methods as per the 'Animals (Scientific Procedures) Act 1986' with an overdose of 2 PE followed by severance of the spinal cord at the base of the skull (Home Office, 1986a; b). Females were distinguished from males by the presence of eggs in their ovaries (all females regardless of reproductive status possess eggs in their ovaries). GSI was calculated for each fish according to section 2.1.2.

2.2. INDIVIDUALS USED TO CONDITION WATER

Fish used to condition water for experiments (donor fish) were housed separately from other individuals of the same sex. Experiments used two independent sources of conditioned water (1) and (2) each containing 10 individuals. Donor fish used to condition water (1) were housed separately from donor fish used to condition water (2). This ensured that the two independent batches of water were conditioned each time using the same individuals (see section 2.4.2 for a description of the protocol used to condition water).

2.3. HOLDING FACILITIES

At CEH, fish were stored in 70 l holding tanks (each containing approximately 5 males and 10 females) under a constant photoperiod (16L: 8D) and room temperature (20 °C - no variation) before the beginning of experiments. Water temperature was maintained at 18 °C. These conditions maintained the normal breeding cycle of the fishes. Holding tanks containing 60 l of water (92 cm long x 31 cm wide x 31 cm high) were arranged in flow through systems each containing a set of three tanks. Water was pumped at a rate of 3 l/min from a reservoir (60 l) below the holding tanks after passing through a gravel filter. Water quality was measured weekly using Nutrafin® aquaria test kits, testing for ammonia, nitrates

and nitrites to ensure that these compounds did not exceed harmful levels. All fish were fed daily with Nutrafin® fish flakes.

To identify the presence of sex pheromones (distinct from a generic response to odour from fish), it was important to test the response to water conditioned by both non reproductive individuals and reproductive individuals (chapters 3, 4 and 5, 6, 7). Non reproductive individuals were held indoors under the same conditions as reproductive individuals for the duration of the winter. Disrupting the natural annual photoperiod and temperature cycle necessary to facilitate a reproductive condition in the wild (Kadmon *et al.*, 1984; Davie *et al.*, 2007 and see a review by Munakata & Kobayashi, 2009), maintained a non reproductive status. GSI measurements were used to confirm their non reproductive status.

2.4. GENERAL EXPERIMENTAL TECHNIQUES

The following experimental techniques were used in chapters 3, 4, 5, 6 and 7.

2.4.1. Preparation of animals for experiments

Before experiments, test animals were fed with Nutrafin® fish flakes to satiation (i.e. an observed cessation of feeding) in order to reduced the likelihood that feeding responses caused changes in activity to conditioned water in the experiments. Experiments were conducted within 1 hour and 15 minutes of feeding.

2.4.2. Conditioning of water

Conditioned water was prepared by placing ten donor fish in a glass container (10 l) containing 6 l of dechlorinated water for 4 hours (Fig. 2.1). As topmouth gudgeon and sunbleak are gregarious (Maekawa *et al.*, 1996; Gozlan *et al.*, 2003a), a large number of donor fish were used in each bowl, rather than singleton fish, to reduce the likelihood that

donor fish release stress related alarm cues (Toa *et al.*, 2004). Two independent sources (using two different groups of 10 donors) of conditioned water were used in trials. Since experimental test tanks were set up in groups of 3, control and conditioned water from one source was sufficient to supply 3 test tanks simultaneously. In total, twelve replicate trials were undertaken using 4 bowls of conditioned water, divided equally between the two different sets of donors (two bowls of water from each set of donor). Chapters 5, 6 and 7 test the hypothesis that responses in females will occur to reproductive male odours isolated via solid phase extraction. Samples for chemical separation and analysis were prepared by housing donor fish in deionised water to avoid interference from components of tap water.

Figure 2.1. Preparation of conditioned water derived from reproductive male topmouth gudgeon *Peusdorasbora parva*. Ten individuals were placed in a 10 l glass container with 6 l of water for 4 hours. Photo by author.

2.4.3. Solid Phase Extraction Procedure

Solid Phase Extraction (SPE) is a separation procedure used to remove compounds from mixtures based on their chemical properties (see Wyatt, 2003). Separation is achieved due to the affinity of solutes in a mixture (the mobile phase) for a solid (the stationary phase) through which the mixture is passed (Supelco, 1998). As sex pheromones in fishes are thought to be hormonal products (steroids and prostaglandins) (Stacey & Sorensen, 2002) which are generally lipophilic and non polar compounds, reversed phase C-18 cartridges have been used successfully as the stationary phase (Zielinski *et al.*, 2003; Miranda *et al.*, 2005; Corkum *et al.*, 2006; Barata *et al.*, 2008). Reversed phase SPE involves a non polar stationary phase and is typically used when the analyte is mid polar to non polar and the mobile phase is polar. This method is therefore applicable for use in separating reproductive chemical cues derived from fishes contained in conditioned water.

In this study, compounds were isolated from conditioned water derived from topmouth gudgeon onto C-18 Solid Phase Extraction (SPE) cartridges (Sigma-Aldrich Company Ltd) by passing the conditioned water (6 l) through 8 SPE cartridges, followed by elution with methanol (5 ml per cartridge) (see Fig. 2.2; 2.3). These samples were dried in a rotary evaporator (Cardiff University) and re-dissolved into one sample using deionised water (5 ml). A control sample was prepared using 6 l of deionised water. Procedure for the preparation of control water SPE isolate was the same as described for conditioned water.

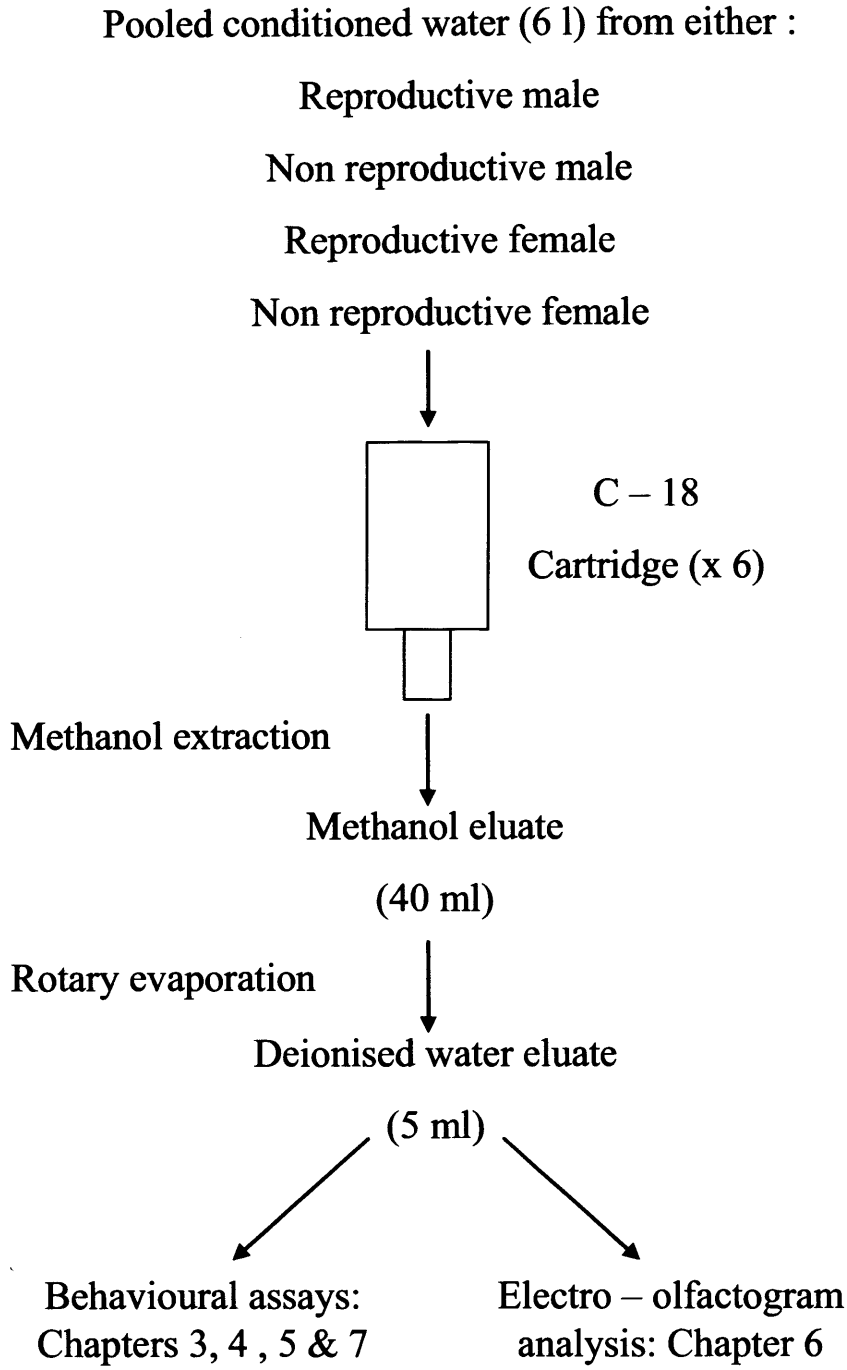

Figure 2.2. Schematic diagram showing the method used for Solid Phase Extraction (SPE) of conditioned water derived from topmouth gudgeon *Pseudorasbora parva*.

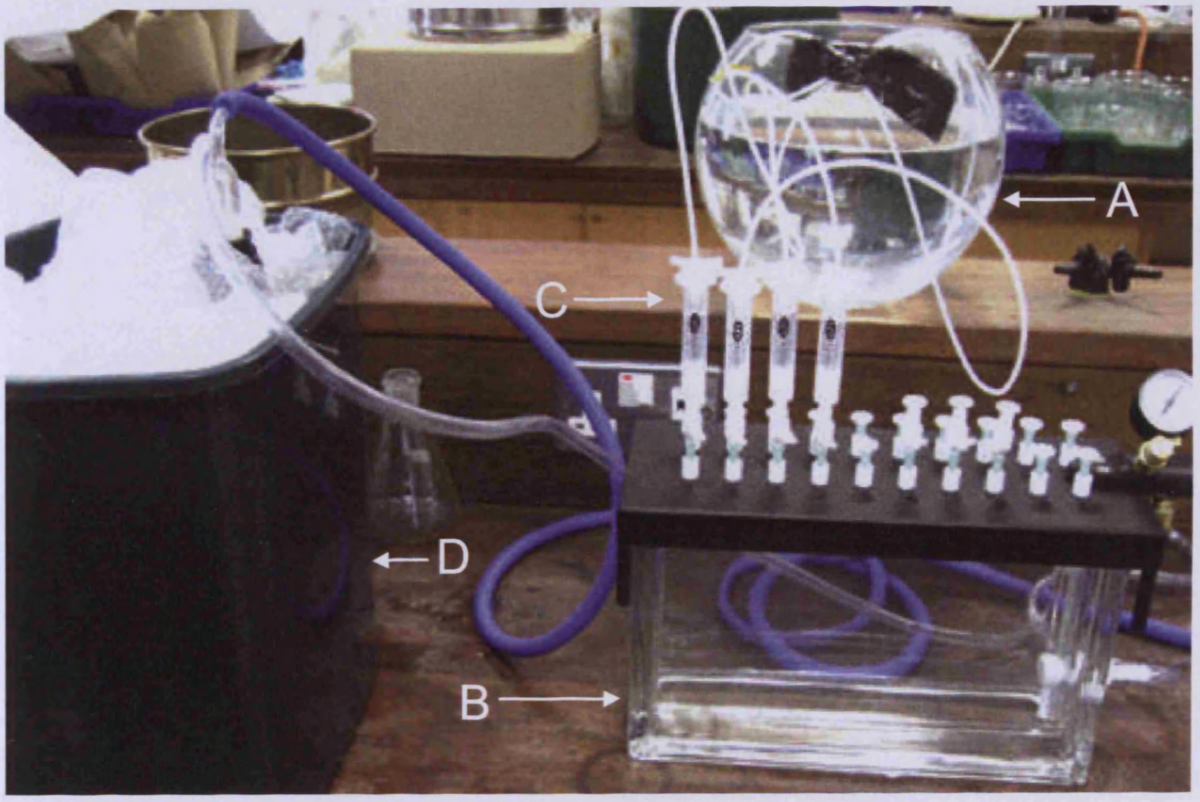

Figure 2.3. Solid Phase Extraction (SPE) experimental design. A = conditioned water, B = SPE manifold, C = C-18 cartridges, D = waste water. Photo by author.

2.4.4. Evaporation using a freeze drier

Conditioned water (6 l) was poured into 6 champagne bottles (1 l per bottle) that had been rinsed thoroughly with deionised water and sterilised in a muffle oven at 300 °C for 8 hours. The samples were then frozen in a -70 °C freezer. Champagne bottles were used in this investigation as they were found to resist shattering upon freezing. Following freezing, the samples were concentrated using a Watson® freeze drier until the bottles were emptied of frozen water (5 days duration). Each bottle was then washed with 45ml deionised water (3 x 15ml washings). These samples were dried in a rotary evaporator (Cardiff University) and re-dissolved into one sample using deionised water (5 ml) (see Fig. 2.4). A blank sample (deionised water) was also prepared. Samples were stored in a -70°C freezer prior to use in experiments (approximately 2 weeks).

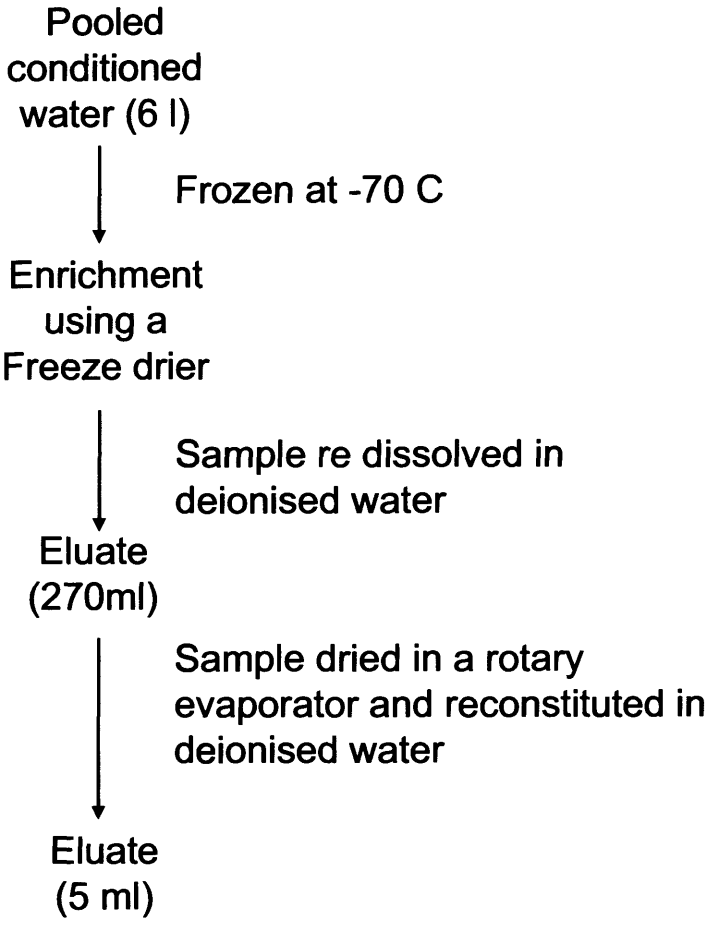

Figure 2.4. Schematic diagram showing the method used for freeze drying conditioned water derived from topmouth gudgeon *Pseudorasbora parva*.

CHAPTER 3. IDENTIFICATION OF A POSSIBLE COURTSHIP RESPONSE TO REPRODUCTIVE MALE CONDITIONED WATER IN FEMALE TOPMOUTH GUDGEON *PSEUDORASBORA PARVA*

3.1. INTRODUCTION

Body postures have long been recognised to have a role in animal communication (Darwin 1872). Many birds (Hinde, 1959), insects (Forsyth & Alcock, 1990) and mammals (Bermant & Davidson, 1974) use visual cues to signal information regarding reproductive receptivity to potential mates. In teleosts, body postures performed during courtship have been documented in species including sunbleak (Gozlan *et al.*, 2003a), three spined stickleback *Gasterosteus aculeatus* L. (ter Pelkwijk & Tinbergen, 1937; Rowland *et al.*, 2002), swordtail *Xiphophorus cortezi* (Gordon) (Fernandez *et al.*, 2008) and Caribbean rosy razorfish *Xyrichtys martinicensis* (Valenciennes) (Victor, 1987).

In teleosts, courtship rituals often involve a head up or head down body posture. In sunbleak a head up vertical position is adopted by reproductive females prior to spawning (Gozlan *et al.*, 2003a). In three spined stickleback a head up (lordosis) posture is indicative of a receptive female (ter Pelkwijk & Tinbergen, 1937). Furthermore, males prefer females that display this signal because in doing so they increase their reproductive success (Rowland *et al.*, 2002). In female swordtail a vertical headstand display is performed during courtship. This specific behaviour is thought to be used by highly receptive females to signal a willingness to mate (Fernandez *et al.*, 2008).

The induction of reproductive behaviour by chemical signals has been well documented in teleosts (see Burnard *et al.*, 2008 for a review). In the best understood species, the goldfish, it is known that inspection behaviour and bouts of chasing amongst males

towards females are induced by the release of Prostaglandin F2α during female surges in luteinising hormone (Kobayashi *et al.*, 2002). In arctic charr *Salvelinus alpinus* L. prostaglandin F2α is released by males and induces female digging behaviour in a chosen nest site (Sveinsson & Hara, 1995). Evidence supporting the initiation of courtship display postures by sex pheromones is more limited. A notable exception, however, is provided by the yellowfish Baikal sculpin *Cottocomephorus grewingki* (Dybowski) where signals released by males induce a courtship 'dance' consisting of quivering body movements in females (Katsel *et al.*, 1992).

In the induction of courtship displays by chemical signals, the sender benefits from the response of the receiver. Here, a potential male can be identified. Individuals that display courting signals benefit by advertising receptivity and therefore increase the chance of attracting a mate. In species such as topmouth gudgeon that use batch spawning as a reproductive strategy and females are at different stages of gonadal development throughout the spawning season (Katona & Maekawa, 1997), display postures adopted by receptive females in response to reproductive male released chemical cues could facilitate successful spawning. This would enable males to discriminate between receptive and non receptive females.

The aim of this study was to identify a specific reproductive response by reproductive females in response to chemical cues released by nest guarding males headstand position has been suggested to demonstrate high receptivity in teleosts species (Fernandez *et al.*, 2008) and may also be used by topmouth gudgeon. Due to the batch spawning reproductive strategy employed by topmouth gudgeon it is hypothesised that a small percentage of reproductive females would adopt a headstand position in response to reproductive male conditioned water.

3.2. MATERIALS AND METHODS

This study was conducted using two experiments. Firstly headstand body postures performed in response to control and stimulus water were recorded. Secondly on identification of a response (a headstand by female) trials were conducted with a larger sample size to determine the proportion of headstand postures within a population (i.e how many females adopt this position).

3.2.1. Experiment one

3.2.2. Experimental design

Three glass tanks (92 cm long x 31 cm wide x 31 cm high) each containing 60 l of dechlorinated tap water were used as test arenas. The bioassay used in this study was based on a design by Colombo *et al.*, (1980). Control and conditioned water was introduced into the test arenas using a peristaltic pump (Watson Marlow® model 323) via Tygon® delivery tubing (5 mm diameter) at a rate of 25 ml/min (Fig. 3.1). Experiments were undertaken under controlled lighting (120 lux - no variation). Test arenas were screened with white laminated paper at the rear and at each side to keep fish in different arenas in visual isolation from one another. Water temperature was maintained at 18 °C (+/- 0.5 °C). Experiments were recorded using 3 ECIR® model KPC-SI90S cameras and a Telexper® TX168 recorder. Cameras were situated 60 cm away from the front of the test arenas.

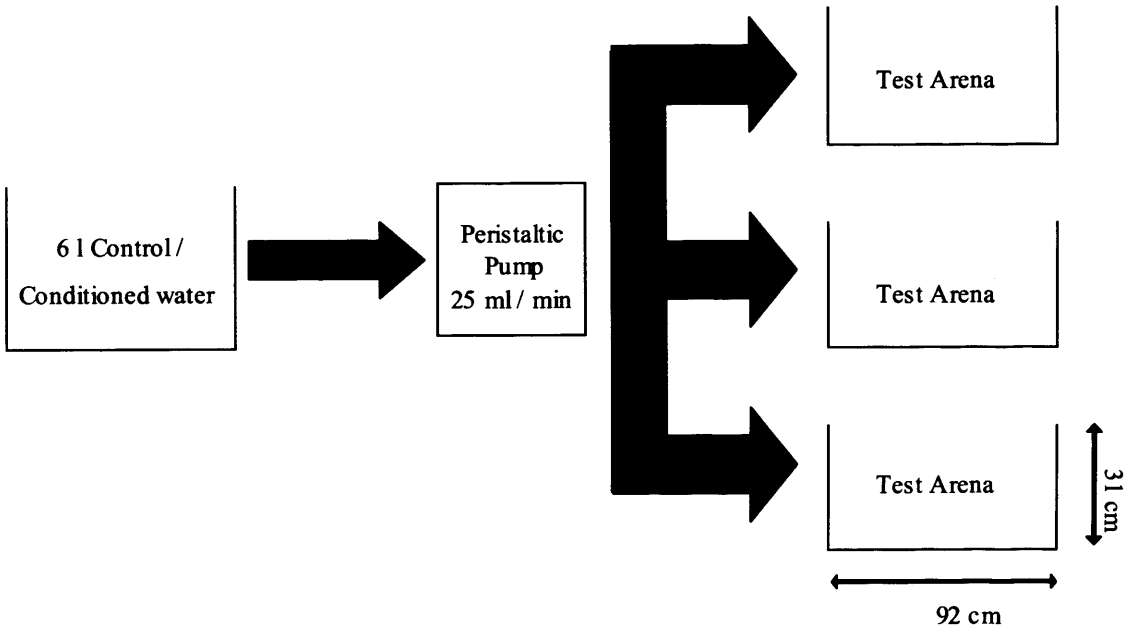

Figure 3.1. Experimental design for testing response to conditioned water (diagram not to scale).

3.2.3. Experimental protocol

In each trial a single individual was exposed to water conditioned by donors (Table 3.1. for combinations, chapter 2, section 2.2 for details on conditioning water and chapter 2, section 2.1.2 for GSI calculations). After 1 hr acclimation period each fish was first exposed to dechlorinated water (control) for 45 min followed by 45 min exposure to stimulus (donor water). Each combination was replicated 12 times using a different fish in each test arena. Experiments were conducted simultaneously in triplicate using a single source of control and stimulus water at a time. No fish was tested more than once. In experimental categories that did not yield a significant response between reproductive donors and reproductive receivers, all other potential experiments (i.e. non reproductive test fish and reproductive donors) in that category were not performed. Headstand body postures performed in response to control and stimulus water were recorded.

Table 3.1. Experiment protocol used to identify headstand courting responses to reproductive chemical cues in topmouth gudgeon *Pseudorasbora parva*. **X** denotes an experiment. Pheromone contributors were donor fish used to condition water. Test fish were studied for their response to conditioned water. In experimental categories that did not yield a response between reproductive donors and reproductive receivers, all other potential experiments in that category were not performed.

			TEST FISH			
			Non reproductive		Reproductive	
			Male	Female	Male	Female
DONOR FISH	Non reproductive	Male		**X**		**X**
		Female				
	Reproductive	Male		**X**	**X**	**X**
		Female			**X**	**X**

3.2.4. Experiment two

Trials were conducted as described in chapter 3, section 3.2.2 with the following exceptions. Smaller test arenas (46cm long x 31 cm wide x 31 cm high) each containing 35 l of dechlorinated tap water were used. This reduced the lag phase before test fish made contact with conditioned water. This reduced the time for each individual trial and ensured that numerous replications could be performed. Control and conditioned water was introduced into the test arenas at a rate of 10 ml/min (see Fig. 3.1). After 1 hr acclimation period each fish was first exposed for 20 min to dechlorinated water (control) followed by 20 min exposure to male conditioned water. A total of 42 females were tested. Headstand body postures were recorded in response to control and reproductive male conditioned water. In addition, swimming activity (the number of horizontal and vertical turns) was quantified in all females that displayed headstands.

3.3. RESULTS

3.3.1. Experiment one

Thirty three percent of reproductive females (n = 12) displayed headstands in the presence of reproductive male conditioned water. Headstands were never observed during control phases or during exposure to non reproductive male or reproductive female

conditioned water. Headstands were not performed by non reproductive females in response to reproductive male conditioned water. Reproductive males did not perform headstands.

3.3.2. Experiment two

Twenty nine percent of females tested (n = 42) displayed headstands in the presence of male conditioned water. No headstands were performed in control phases. Individual females displayed up to seven discrete headstands but the first headstand always occurred within the first 12 minutes of the bioassays (Fig. 3.2). Fewer fish performed headstands in the second half of the stimulus period (n = 4) than the first half (n = 11) There was a significant increase in swimming activity (n = 12, P = < 0.01) (Fig. 7.2) between control and stimulus periods in all fish that performed headstands (Fig 3.3).

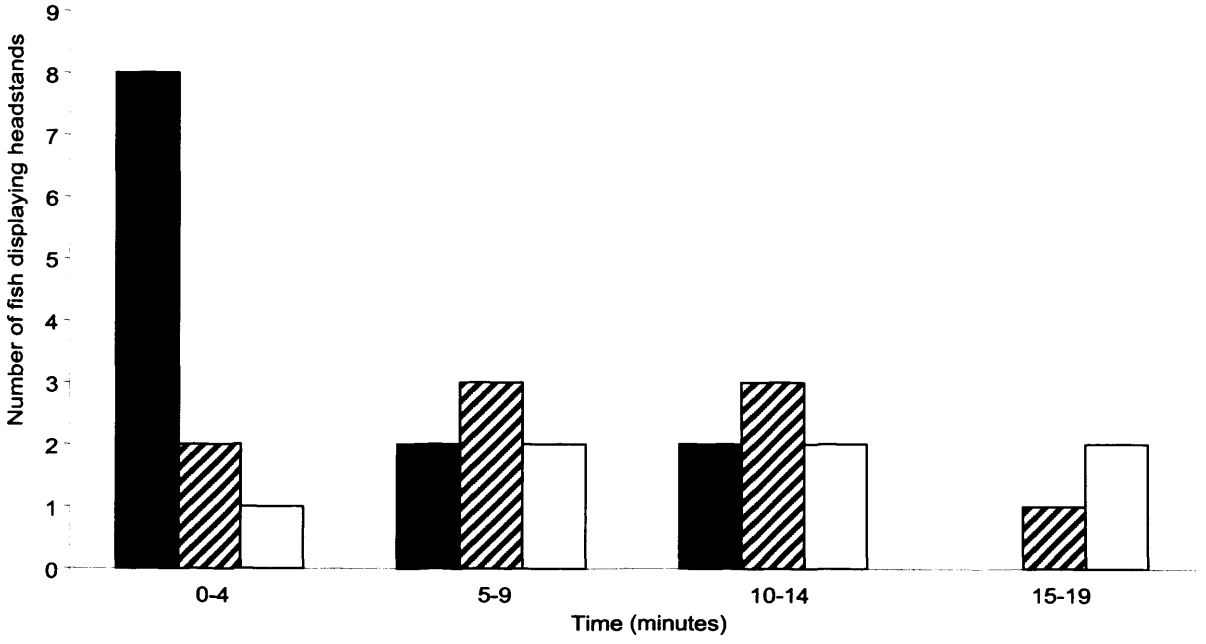

Figure 3.2. The number of reproductive female topmouth gudgeon *Pseudorasbora parva* displaying their first (solid), second (lined) and third or more (clear) headstand courtship postures when exposed to water conditioned by reproductively active males (n = 12). Data are presented separately for each of the time periods within the total 20 minute exposure period.

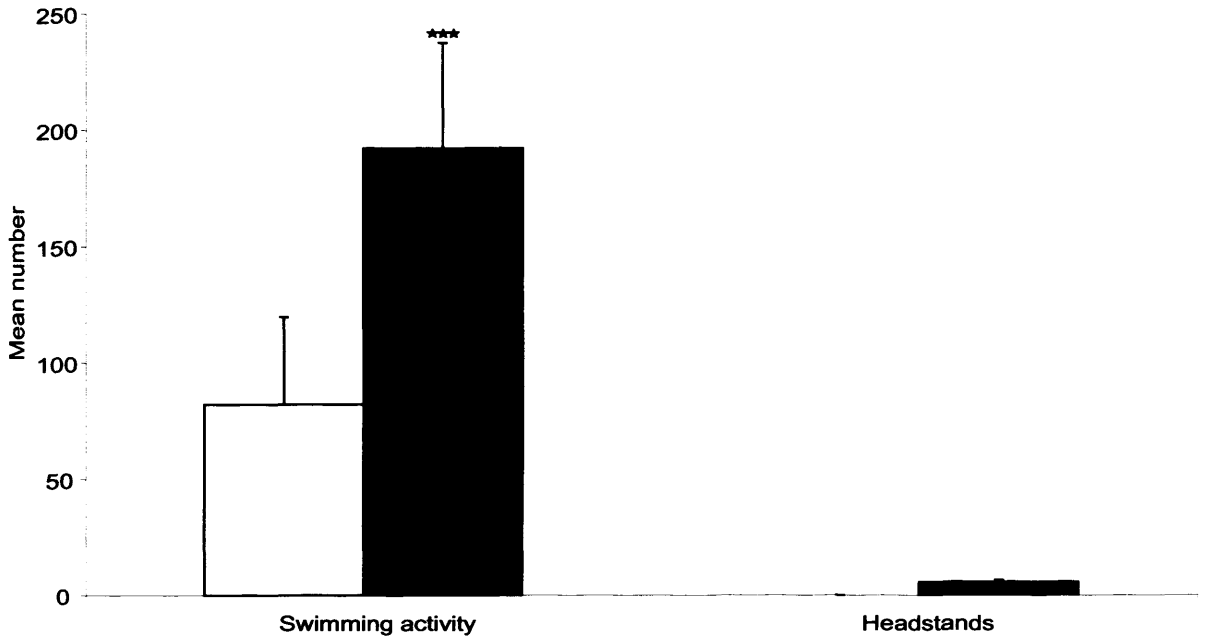

Figure 3.3. Mean number of swimming turns and headstands performed by reproductive female topmouth gudgeon *Pseudorasbora parva* displaying headstands (n = 12) during exposure to control (clear bars) and reproductive male conditioned water (solid bars). Swimming activity is defined as the sum of vertical and horizontal turns. Data are presented from the total 40 minute experiment time (control = 20 min, stimulus = 20 min). *** represents *P*-values below 0.005. Error bars represent standard error.

Mean GSI of reproductive male topmouth gudgeon (mean total length 7 cm, S.D. 1.1 cm; mean weight 6.5 g, S.D. 1.0 g, n = 60) that were used to condition water in this chapter were compared with a group of non reproductive males (mean total length 7.3 cm, S.D. 0.8 cm; mean weight 6.0 g, S.D. 1.2 g, n = 40) that were used as test fish in chapter 3. Mean GSI of reproductive and non reproductive males was 3.7 (S.D. 1.3) and 1.1 (S.D. 0.5) respectively. There was a significant difference in the GSI between these two groups of males (Mann-Whitney *U* Test, $P < 0.001$). Mean GSI of reproductive female (mean total length 5.2 cm, S.D. 1.0 cm; mean weight 1.5 g, S.D. 0.4 g, n = 78) were compared with a group of non reproductive female topmouth gudgeon (mean total length 5.6 cm, S.D. 1.1 cm; mean weight 1.6 g, S.D. 0.2 g, n = 24) that were used as test fish in chapter 3. Mean GSI of reproductive and non reproductive females was 13.5 (S.D. 6.4) and 6.7 (S.D. 3.0) respectively. There was a significant difference in the GSI between these two groups of females (Mann-Whitney *U*

Test, $P = < 0.001$). These results show that visual identification was an accurate measure of reproductive condition.

3.4. DISCUSSION

The results of this study suggest that reproductive females perform a visual signal in response to reproductive male released chemical signals. Overall in this investigation, 28 % of reproductive females performed headstands in response to reproductive male conditioned water only. While, by comparison, no headstands were performed by males. As reproductive females did not perform headstands in response to water conditioned by donors other than reproductive males, and non reproductive females did not perform headstands in response to reproductive males, the findings of this chapter suggest that a headstand posture could have a reproductive function in topmouth gudgeon that is induced by male released signals.

In the swordtail, only a small percentage of reproductive females assumed a headstand stance in the presence of males suggesting that the posture is a visual signal indicating a readiness to mate (Fernandez *et al.*, 2008). Similarly, in this study the percentage of reproductive females that performed headstands during exposure to male conditioned water was low (approximately a third) suggesting that headstand behaviour could be a visual cue that signals high receptivity to potential mates in topmouth gudgeon. As topmouth gudgeon are batch spawners and therefore individuals are in different stages of gonadal maturity during the reproductive season and only 70% actually lay eggs (Katona & Maekawa, 1997) it is expected that the frequency of individuals displaying a receptivity signal would be low within a population at any given time.

In this study, the chance of a female displaying headstand courtship behaviour decreased rapidly after 5 minutes exposure to conditioned water. There are two possible explanations for this. Firstly, as the test arenas were not flow through systems there was an

increasing concentration of male derived substances in the test tanks that could have resulted in sensory adaptation. Secondly, cessation of female headstand postures in the absence of males demonstrates the need for visual cues in the mating process. As headstands did occur in 3 fish after 10 minutes exposure to stimulus, sensory adaptation is an unlikely explanation, this would require a cessation of headstands in all fish (as adaptation of olfactory receptors would occur in all fish). Seventy five percent of displaying females had performed courtship behaviour within 5 minutes of exposure time; therefore the results of this chapter suggest a refined bioassay with a reduced trial period can be used for further study.

As courtship behaviour in topmouth gudgeon has not been well documented, it is not possible to definitively conclude that the observed headstands in response to reproductive male conditioned water are used as a signal during mating. However, a headstand display stance has been documented to occur in other teleosts during courtship (Gozlan *et al.*, 2003a; Fernandez *et al.*, 2008) and only occurs during exposure to reproductive male conditioned water in the test species. This suggests that the headstand posture does have a role in the courting behaviour of topmouth gudgeon. The stage of female fertility could not be determined in this study, therefore it is not possible to conclude that headstand behaviour is a signal of receptivity. To determine conclusively whether headstands signal high receptivity, egg histology or hormone levels throughout the spawning season would need to be examined.

In accordance with other species that demonstrate male paternal care notably the round goby (Belanger *et al.*, 2004) and sea lamprey (Li *et al.*, 2002) reproductive female topmouth gudgeon increased their swimming activity (the number of horizontal and vertical turns) during exposure to reproductive male conditioned water. Further research is required to determine if this response occurs only in reproductive females (not in non reproductive females) and only during exposure to cues released by reproductive males (not non

reproductive males) in the study species (Chapter 4). This would provide further evidence of reproductive chemical communication in the study species.

This study shows that behaviour performed by females during courtship in some teleost species is observed during exposure to a signal released by reproductive males. This provides an example of chemical communication in a teleost that has a distinct pheromone system from that of the most understood model, the goldfish (see Kobayashi *et al.*, 2002 for a review). In topmouth gudgeon, chemical signals released by males induce behavioural responses in females contrasting reproductive chemical communication in the goldfish where responses in males are induced by female released cues (Sorensen *et al.*, 1990). This provides preliminary evidence that sex pheromone systems have evolved with reproductive strategy. The great variation in both mating strategy and courting behaviour in fishes suggests that sex pheromone systems would also be diverse and further study is required before the goldfish model should be regarded as a blueprint for reproductive chemical communication in teleosts.

CHAPTER 4. IDENTIFICATION OF A CONSPECIFIC RESPONSE TO CONDITIONED WATER IN TOPMOUTH GUDGEON *PSEUDORASBORA PARVA* AND SUNBLEAK *LEUCASPIUS DELINEATUS*

4.1. INTRODUCTION

Since the identification of a reproductive chemical cue in the black goby *Gobius niger* L. (Colombo *et al.*, 1980), the list of freshwater fish species that show evidence for use of sex pheromones has grown considerably (Burnard *et al.*, 2008). Sex pheromones are involved in a diverse range of interactions but the responses they evoke in fishes can be divided into primer and releaser types (Stacey & Sorensen, 2006). Primer responses include gonadal development or hormonal changes due to exposure to isolated compounds (Moore & Waring, 1996) or unpurified conspecific odours (Olsen *et al.*, 2000). In goldfish for example, 17,20β-Progesterone induces an increase in milt volume and motility (see Kobayashi *et al.*, 2002 for a review). In fish smaller than the goldfish, however such as topmouth gudgeon and sunbleak, milt extraction is difficult (Personal communication - R.E. Gozlan; P.C. Hubbard) therefore accurate measurement of these described parameters is currently challenging. In such small-bodied fish, responses to sex pheromones are currently limited to releaser responses.

Releaser responses are defined as the triggering of reproductive behaviour during exposure to isolated compounds (Laberge & Hara, 2003) or unpurified conspecific odour (Serrano *et al.*, 2008). Documented examples include male spawning behaviour in the goldfish (see Kobayashi *et al.*, 2002) and female attraction in Arctic Charr (Sveinsson & Hara 1995). There are numerous examples of behavioural responses that are exhibited by males on

exposure to chemical cues released by females (see Burnard *et al.,* 2008) but in some species, e.g. round gobies where males guard nests, chemical cues produced by males induce behavioural responses in females (Belanger *et al.*, 2004). Studies concerning releaser responses may be particularly important to fisheries managers and conservation biologists facing the problem of invasive species as reproductive behaviour can be exploited e.g. to facilitate capture of individuals via pheromone traps (Corkum *et al.*, 2007).

The large diversity of reproductive behaviour apparent in different species of freshwater fish (see chapter 1, table 1.1) makes the identification of specific responses to pheromones challenging. Reliable bioassay development that permits accurate identification of specific behaviours is difficult without prior knowledge of the full range of behaviour (Bentley & Watson, 2000). Furthermore, specific responses to reproductive chemical cues often only occur in receptive individuals (see chapter 3) and in species such as topmouth gudgeon and sunbleak where females are at different stages of gonadal development throughout the spawning season, responses may be restricted to a short time period. Quantifying a generic response such as an increase in swimming activity to water-borne sex pheromones is however a feasible option allowing identification of responsive species. An increase in swimming activity implies recognition of a relevant chemical cue.

The aim of this investigation was to study a generic response to conspecific conditioned water in topmouth gudgeon and sunbleak. In both species, males guard nests in which females lay eggs (Maekawa *et al.*, 1996; Gozlan *et al.*, 2003a). It was therefore hypothesised that reproductive female topmouth gudgeon and sunbleak would increase their swimming activity (the number of turns) and vagility (inherent tendency to move) in response to conspecific reproductive male conditioned water. Fishes were exposed to conditioned water and not to individual fractions isolated from conditioned water via e.g. solid phase extraction

techniques to avoid partial or total loss of activity due to incomplete or lack of recovery. Conditioned water contains all the potential compounds that might elicit a response.

4.2. MATERIALS AND METHODS

4.2.1. Experimental design

Experiments were conducted using the design described in chapter 3, section 3.2.2 with the following exception. Each arena was divided into 12 equal squares using lines drawn on the glass sides (front and rear).

4.2.2. Experimental protocol

In each trial a single individual was exposed to water conditioned by donors (see, chapter 2, section 2.2 for details on conditioning water and chapter 2, section 2.1.2 for GSI calculations). After 1 hr acclimation period each fish was first exposed to dechlorinated water (control) for 45 min followed by 45 min exposure to stimulus (donor water). Each combination was replicated 12 times using a different fish in each test arena. Experiments were conducted simultaneously in triplicate using a single source of control and stimulus water at a time. No fish was tested more than once. Fish activity and vagility was measured using the behavioural program ethолog® (Ottoni, 2000). Swimming activity was represented by the number of changes in horizontal and vertical swimming directions. A change in direction of 180° was considered as a turn (Laberge & Hara, 2003). Swimming vagility (inherent tendency to move) was represented by the number of squares entered (partitioning each test tank). The total number of turns and the total number of times each square was entered was quantified. The total duration of time spent in each half of the tank (squares 1, 2, 3, 7, 8, 9 and 4, 5, 6, 10, 11, 12) was also quantified for the first 15 minutes when the system was not saturated with treatment water (confirmed by dye runs). A Wilcoxon signed ranks test was used to analyse data. In experimental categories that did not yield a significant response

between reproductive donors and reproductive receivers, all other potential experiments (i.e. non reproductive test fish and reproductive donors) in that category were not performed.

4.3. RESULTS

Reproductive female topmouth gudgeon and reproductive female sunbleak responded to cues released by conspecific reproductive males. In addition, reproductive male sunbleak responded to cues released by reproductive females and reproductive males. In topmouth gudgeon this response was shown by a significant increase in swimming vagility (Wilcoxon signed ranks test) between control (dechlorinated tap water) and stimulus (conditioned water) when reproductive females were exposed to water conditioned by reproductive males (Table 4.1). In sunbleak there were significant increases in swimming vagility (Wilcoxon signed ranks test) when reproductive males were exposed to water conditioned by reproductive males and when reproductive females were exposed to water conditioned by reproductive males (Table 4.1).

Table 4.1. *P*-values for swimming vagility (inherent tendency to move) in response to conspecific conditioned water in topmouth gudgeon *Pseudorasbora parva* and sunbleak *Leucaspius delineatus* (n = 12). Bold denotes significant response. All significant responses represent an increase in swimming vagility except * which represents a decrease in swimming activity.

				TEST FISH							
				Topmouth Gudgeon				Sunbleak			
				Non reproductive		Reproductive		Non reproductive		Reproductive	
				Male	Female	Male	Female	Male	Female	Male	Female
DONOR FISH	Topmouth Gudgeon	Non reproductive	Male		0.57		0.07*				
			Female								
		Reproductive	Male		0.70	0.48	**<0.01**				
			Female			0.43	0.94				
	Sunbleak	Non reproductive	Male					0.75	0.81	0.99	0.790
			Female					0.70		0.48	
		Reproductive	Male					0.94	0.64	**<0.05**	**<0.05**
			Female					0.53		0.18	0.21

In topmouth gudgeon there was a significant increase in swimming activity (Wilcoxon signed ranks test) between control (dechlorinated tap water) and stimulus (conditioned water) when reproductive females were exposed to water conditioned by reproductive males (Table 4.2). In sunbleak there were significant increases in swimming activity when reproductive males were exposed to water conditioned by reproductive females, reproductive males and when reproductive females were exposed to water conditioned by reproductive males (Table 4.2).

Table 4.2. *P*-values for swimming activity (pooled horizontal and vertical turns) in response to conspecific conditioned water in topmouth gudgeon *Pseudorasbora parva* and sunbleak *Leucaspius delineatus* (n = 12). Bold denotes significant response. All significant responses represent an increase in swimming activity.

				TEST FISH							
				Topmouth Gudgeon				Sunbleak			
				Non reproductive		Reproductive		Non reproductive		Reproductive	
				Male	Female	Male	Female	Male	Female	Male	Female
DONOR FISH	Topmouth Gudgeon	Non reproductive	Male		0.75		0.21				
			Female								
		Reproductive	Male		0.39	0.99	**<0.01**				
			Female			0.53	0.64				
	Sunbleak	Non reproductive	Male					0.84	0.78	0.88	0.88
			Female					0.67		0.72	
		Reproductive	Male					0.94	0.58	**<0.01**	**<0.01**
			Female					0.27		**<0.01**	0.61

In this study, increases in swimming activity and vagility were not coupled with attraction to the odour source in either study species. There were no significant differences between time spent in the half of the tank close to the odour source (squares 1, 2, 3, 7, 8, 9) during the first 15 minutes between control and stimulus phases when reproductive female topmouth gudgeon were exposed to reproductive male water (Wilcoxon signed ranks test, P = 0.793). There were also no significant differences between time spent in the half of the tank close to the odour source between control and stimulus phases during the first 15 minutes when reproductive female sunbleak were exposed to reproductive male conditioned water (P

= 0.492) or when reproductive males were exposed to reproductive female (P = 0.730) or male conditioned water (P = 0.635).

Mean GSI of reproductive male (mean total length 7 cm, S.D. 1.1 cm; mean weight 6.5 g, SD 1.0 g, n = 64) and non reproductive male (mean total length 7.3 cm, S.D. 0.8 cm; mean weight 6.0 g, S.D. 1.2 g, n = 40) topmouth gudgeon used in this study was 2.7 (S.D. 1.1) and 1.1 (S.D. 0.5) respectively. There was a significant difference in the GSI between these two groups of males (Mann-Whitney U Test, $P < 0.001$). Mean GSI of reproductive female (mean total length 5.3 cm, S.D. 0.8 cm; mean weight 1.6 g, S.D. 0.6 g, n = 36) and non reproductive female topmouth gudgeon (mean total length 5.6 cm, S.D. 1.1 cm; mean weight 1.6 g, S.D. 0.2 g, n = 24) used in this study was 15.4 (S.D. 7.5) and 6.7 (S.D. 3.0) respectively. There was a significant difference in the GSI between these two groups of females (Mann-Whitney U Test, $P < 0.001$). These results show that visual identification was an accurate measure of reproductive condition.

Mean GSI of reproductive male sunbleak (mean total length 5.5 cm, S.D. 0.6 cm; mean weight 1.9 g, S.D. 0.4 g, n = 88) and non reproductive male sunbleak (mean total length 5.3 cm, S.D. 0.6 cm; mean weight 1.7 g, S.D. 0.7 g, n = 88) used in this study was 4.2 (S.D. 0.5) and 2.6 (S.D. 0.3) respectively. There was a significant difference in the GSI between these two groups of males (Mann-Whitney U Test, $P < 0.001$). Mean GSI of reproductive female (mean total length 5.9 cm, S.D. 0.6 cm; mean weight 1.9 g, S.D. 0.5 g, n = 76) and non reproductive female sunbleak (mean total length 5.7 cm, S.D. 1.1 cm; mean weight 1.6 g, S.D. 0.2 g, n = 64) used in this study was 15.4 (S.D. 7.5) and 6.7 (S.D. 3.0) respectively. There was a significant difference in the GSI between these two groups of females (Mann-Whitney U Test, $P < 0.001$). These results show that visual identification was an accurate measure of reproductive condition.

4.4. DISCUSSION

The results presented in this study show that reproductive chemical cues operate in both topmouth gudgeon and sunbleak suggesting the presence of a sex pheromone system in both species. In reproductive female topmouth gudgeon and sunbleak an increase in both swimming activity and vagility in response to conspecific reproductive male conditioned water confirms the presence of male released chemical cues. In reproductive male sunbleak an increase in both swimming activity and vagility was identified in response to reproductive female and reproductive male released chemical cues.

In this study, there was no increase in swimming activity or vagility in response to conspecific conditioned water when both sender (donors) and receiver (test individuals) were not in a reproductive condition. This shows that responses when both sexes are reproductively active are linked to the reproductive condition of the fishes. In accordance with previous studies (Belanger *et al.*, 2004; Corkum *et al.*, 2006) only reproductive fishes responded to water conditioned by reproductive donors showing that information provided by the chemical cue is relevant only to individuals in a reproductive state. As reproductive chemical cues provide information on reproductive status to potential receivers, it can be expected that only females that are in a reproductive status and would be receptive (or nearing receptivity) to males would respond to these chemical cues. The bioassay used in this study was not designed to identify a specific reproductive response to chemical cues; so it is not possible to conclude definitively that a chemical communication system is in operation in these species (see chapter 7). Here an evolved response to the cue (by the receiver) could signal reproductive information (for example a willingness to mate) to the original sender (Stacey & Sorensen, 2006).

In accordance with previous studies (Haberli & Aeschlimann, 2004; Gammon *et al.*, 2005) that show female olfactory preference for reproductive males over non reproductive

males, in this study responses occur only to water conditioned by reproductive active donors. This shows that the chemical cues are only released by individuals in a reproductive state. As sex pheromone systems are hypothesised to have evolved by 'spying' (the chance expression of hormone receptors on olfactory tissue) metabolic products released by individuals that are in a reproductive condition (Sorensen & Stacey, 2004), it can be expected that responses to chemical cues only occur to water conditioned by reproductive individuals that release these products.

In the goldfish, release of a female preovulatory pheromone that induces courtship behaviour in males is released post vitellogenesis. This pheromone comprises of a mixture that includes Prostaglandin F2α, the hormone that regulates and induces female sexual behaviour (see Kobayashi *et al.*, 2002 for a review). This current study was not designed to determine the timing of the release of chemical cues, however as males build nests in both topmouth gudgeon and sunbleak, it can be hypothesised that cues are released after nest completion. This would coincide with a period during which males are searching for females to lay eggs in their nest. Further research is required to investigate the relationship between internal hormone levels and pheromone production and release.

In addition to a response by reproductive males to water conditioned by reproductive females, a response to water conditioned by other reproductive males was also identified in sunbleak. Whilst the precise functionality underlying this response was not identified here with certainty, it is possible to speculate that chemical cues released by reproductive males are important signals to conspecific males. Sunbleak have a reproductive strategy based on allopaternal care where males guard the eggs of previous inhabitants in a communal nest (Gozlan *et al.*, 2003a). In fathead minnows (that also use a form of allopaternal care) males evict previous males from nest sites and care for their eggs (Unger & Sargent, 2004). It could be expected that given the reproductive strategy employed by

sunbleak, cues released by males are important to conspecific males, possibly to inform the receiver of the reproductive status of the nest guarder.

Despite the fact that the bioassay used in this study was designed to identify a generic response to conditioned water (thus providing evidence for the existence of chemical communication) the responses of test fishes does allow for some observations to be made regarding specific functions of the released cues. In the female sea lamprey and the female round goby, exposure to reproductive male conditioned water induced an increase in swimming activity (Li *et al.*, 2002; Belanger *et al.*, 2004; Gammon *et al.*, 2005). Specifically, in the sea lamprey, pooled components of swimming activity (swimming back and forth, tail beating and swimming speed) was significantly greater in response to male conditioned water than when exposed to control water (Li *et al.*, 2002: Siefkes *et al.*, 2003). This response is indicative of searching behaviour (Li *et al.*, 2002). The observed increase in swimming activity and vagility during exposure to conditioned water evoked in the test species in this current investigation is in accordance with these findings. The results strongly suggest that releaser type chemical cues are in operation in both test species. However, the current experiments would need to be replicated in a flow-through system using only one donor fish to conclude that the observed responses are indicators of searching behaviour. Here, experimental conditions would more accurately replicate the conditions of a natural system using a constant concentration of derived odour from just one fish, as opposed to an increasing concentration that derived from multiple fish.

In the sea lamprey, (Li *et al.*, 2002: Siefkes *et al.*, 2003), black goby (*Colombo et al.*, 1980) and round goby (Gammon *et al.*, 2005) induced searching behaviour was also coupled with attraction to the source of conditioned water. This is proposed to guide females to the nest site (Li *et al.*, 2002, Corkum *et al.*, 2006). In this current investigation, there was no attraction to the source of conditioned water in either test species. This may be related to

the differing reproductive strategy of primitive nest guarders (such as topmouth gudegon and sunbleak tested in this study: Maekawa *et al.*, 1996; Brezeanu *et al.*, 1968; Gozlan *et al.*, 2003a) compared to the true nest guarding behaviour of other studied species (Mozzi, 1978; Wickett & Corkum, 1998; Li *et al.*, 2003). In true nest guarders, males do not leave the nest but instead females are enticed into it (see Wickett & Corkum, 1998; Li *et al.*, 2003). Therefore it could be expected that male released cues would be used by females to locate nest sites. In both topmouth gudgeon and sunbleak, males leave the nest site to search for females (Maekawa *et al.*, 1996; Gozlan *et al.*, 2003a). Therefore it could be expected that females would not be attracted to the source of conditioned water since the male does not stay in one specific place but instead roams across an area. However, induced searching behaviour in females on recognition of the cue could still occur as the odour signifies that a male is close by. As previously stated attraction to a point source would need to be tested in a functional bioassay, specifically a flow-through system. It is worth adding however, that attraction to the source of conditioned water was documented in the black goby using a closed system (Colombo *et al.*, 1980).

In this current investigation, there was no observed behavioural response in male topmouth gudgeon to cues released by reproductive females or reproductive males. This is in accordance with the behaviour observed by male round gobies during exposure to reproductive male and reproductive female odours (Marentette & Corkum, 2008). In the round goby a lack of behavioural response could be expected as males do not search for females. However in topmouth gudgeon, males do search for females (Maekawa *et al.*, 1996), therefore it is surprising that there is no apparent behavioural response in reproductive males to reproductive female conditioned water. It is possible that as the bioassay did not incorporate a nest site and therefore the males was not actively guarding a nest, behavioural responses were not induced. Furthermore, in agreement with Marentette & Corkum, (2008) a

lack of behavioural response in males does not mean that female reproductive cues do not have a role in reproductive chemical communication. It is possible, for example, that physiological changes (primer effects) occur due to exposure to a chemical cue that may not necessarily result in discriminatory behaviour.

Responses to male released chemical cues have been identified in species where the male guards nests (Belanger *et al.*, 2004). Similarly in this study, a response to male conditioned water by reproductive females was identified in two species that employ male nest guarding. In some species, such as the goldfish, courtship behaviour is induced by chemical cues (see Kobayashi *et al.*, 2002) and in others such as the round goby, concentration gradients lead recipients to nest sites (Belanger *et al.*, 2004). Further study should aim to investigate the link between the response to conditioned water and the reproductive function that causes this response in functional bioassays providing more conclusive evidence of operating sex pheromone systems.

CHAPTER 5. THE RECOGNITION OF HETEROSPECIFIC REPRODUCTIVE CHEMICAL CUES IN TOPMOUTH GUDGEON *PSEUDORASBORA PARVA* AND SUNBLEAK *LEUCASPIUS DELINEATUS*

5.1. INTRODUCTION

In reproductive chemical communication systems discrimination between multi-component pheromone 'blends' prevents interspecific breeding (see Wyatt, 2003). Here, more than one compound is required to facilitate reproduction and different species have different mixtures (blends) of compounds preventing successful mating. For insects this phenomenon has been well studied (see chapter 1). Sorensen & Scott, (1994) suggest that the use of steroidal and prostaglandin based compounds as sex pheromones is widespread amongst freshwater teleosts, therefore discrimination between different components of sex pheromones is also likely to occur. It is therefore possible that recognition and response to components of a sex pheromone occur but successful courtship is prevented by the absence of another compound. In the allopatric species Montezuam swordtail *Xiphophorus montezumae* (Jordon and Snyder) and the Panuco swordtail *Xiphophorus nigrenisis* (Rosen), females preferred heterospecific odours over control water, but preferred conspecific odour when matched against heterospecific odour (Mclennan & Ryan, 1999).

It is known that sex pheromone systems in fish species that hybridise are sometimes similar (Sorensen *et al.*, 1998). In brown trout and Atlantic salmon lack of species specificity to primer cues results in elevated plasma levels of hormones that are associated with reproductive behaviour. Specifically, increased testosterone, 11 keto-testosterone and 17α,20β-dihydroxy-4-pregnen-3-one occurs in response to conspecific and heterospecific

odours (Olsen *et al*, 2000). It is also known, however, that the same pheromone can evoke different responses. For example conjugated (sulphated) 17α,20β-dihydroxy-4-pregnen-3-one evokes inspection of females by male goldfish (Kobayashi *et al.*, 2002) but increases milt production in hill trout (Bhatt & Sajwan, 2001).

Intraspecific recognition of relevant reproductive cues has been shown in the species topmouth gudgeon and sunbleak (chapter 3). However the question of whether these cues are recognised only by conspecifics or also by heterospecifics remains untested. Both species have similar reproductive strategies. In particular, male nest guarding is common to both species. In this chapter the hypothesis that recognition of reproductive chemical cues occurs between topmouth gudgeon and sunbleak is tested. A behavioural response in reproductive fishes to water conditioned by reproductive donors would imply that the signal is relevant to the reproductive condition of the receiver.

5.2. MATERIALS AND METHODS

5.2.1. Experimental design

Experiments were conducted as described in chapter 4, section 4.1.

5.2.2. Experimental protocol

In each trial a single individual was exposed to water conditioned by heterospecific donors (chapter 2 section 2.2 for details on conditioning water and chapter 2, section 2.1.2 for GSI calculations). Experiments were conducted as described in chapter 4, section 4.2. Swimming activity (the number of horizontal and vertical turns) and swimming vagility (total number of times each square was entered) was quantified (see chapter 4, section 4.2.2 for details). A Wilcoxon signed ranks test was used to analyse data. In experimental categories that did not yield a significant response between reproductive donors and reproductive

receivers, all other potential experiments (i.e. non reproductive test fish and reproductive donors) in that category were not performed.

5.3. RESULTS

The results show that reproductive male and female sunbleak respond to cues released by reproductive topmouth gudgeon. Specifically, in reproductive male sunbleak there was a significant increase in both swimming vagility and activity in response to odour from reproductive male and female topmouth gudgeon (Tables 5.1, 5.2). In reproductive female sunbleak there was a significant increase in both swimming vagility and activity in response to reproductive male topmouth gudgeon conditioned water. Sunbleak only responded to cues released by reproductive topmouth gudgeon. There were no significant increases in swimming vagility or activity when reproductive sunbleak were exposed to water conditioned by non reproductive topmouth gudgeon. Only reproductive sunbleak responded to cues released by reproductive topmouth. Reproductive female sunbleak did not respond to cues derived from reproductive female topmouth gudgeon. There were no significant increases in swimming vagility or activity when non reproductive sunbleak were exposed to water conditioned by reproductive topmouth gudgeon. Topmouth gudgeon did not respond to odours derived from sunbleak. There was no significant increase in swimming vagility or activity when reproductive topmouth gudgeon were exposed to water conditioned by reproductive sunbleak.

Table 5.1. *P*-values for swimming vagility in response to heterospecific conditioned water in topmouth gudgeon *Pseudorasbora parva* and sunbleak *Leucaspius delineatus* (n = 12). Bold denotes significant response. All significant responses represent an increase in swimming vagility.

				TEST FISH							
				Topmouth Gudgeon				Sunbleak			
				Non reproductive		Reproductive		Non reproductive		Reproductive	
				Male	Female	Male	Female	Male	Female	Male	Female
DONOR FISH	Topmouth Gudgeon	Non reproductive	Male					0.31	0.64	0.35	0.97
			Female					0.79		0.48	
		Reproductive	Male					0.69	0.97	**<0.05**	**<0.01**
			Female					0.40		**<0.05**	0.93
	Sunbleak	Non reproductive	Male								
			Female								
		Reproductive	Male			0.21	0.58				
			Female			0.59	0.27				

Table 5.2. *P*-values for swimming activity in response to heterospecific conditioned water in topmouth gudgeon *Pseudorasbora parva* and sunbleak *Leucaspius delineatus* (n = 12). Bold denotes significant response. All significant responses represent an increase in swimming activity.

				TEST FISH							
				Topmouth Gudgeon				Sunbleak			
				Non reproductive		Reproductive		Non reproductive		Reproductive	
				Male	Female	Male	Female	Male	Female	Male	Female
DONOR FISH	Topmouth Gudgeon	Non reproductive	Male					0.12	0.12	0.31	0.53
			Female					0.39		0.08	
		Reproductive	Male					0.75	0.94	**<0.05**	**<0.01**
			Female					0.53		**<0.01**	0.81
	Sunbleak	Non reproductive	Male								
			Female								
		Reproductive	Male			0.16	0.70				
			Female			0.16	0.39				

The mean GSI of reproductive male topmouth gudgeon (mean total length 7.4 cm, S.D. 1.5 cm; mean weight 6.9 g, S.D. 0.7 g, n = 64) and non reproductive male topmouth gudgeon (mean total length 7.0 cm, S.D. 0.4 cm; mean weight 6.2 g, S.D. 1.1 g, n = 40) used in this study was 2.9 (S.D. 1.5) and 1.0 (S.D. 0.5) respectively. There was a significant difference in the GSI between these two groups of males (Mann-Whitney *U* Test, $P < 0.001$). Mean GSI of reproductive female (mean total length 5.0 cm, S.D. 0.7 cm; mean weight 1.9 g,

S.D. 0.8 g, n = 64) and non reproductive female topmouth gudgeon (mean total length 4.9 cm, S.D. 0.4 cm; mean weight 1.2 g, S.D. 0.5 g, n = 64) used in this study was 19.2 (S.D. 7.8) and 4.9 (S.D. 2.0) respectively. There was a significant difference in the GSI between these two groups of females (Mann-Whitney *U* Test, $P < 0.001$). These results show that visual identification was an accurate measure of reproductive condition.

The mean GSI of reproductive male sunbleak (mean total length 5.3 cm, S.D. 0.6 cm; mean weight 2.1 g, S.D. 0.7 g, n = 68) used in this study was compared with a group of non reproductive males (mean total length 5.3 cm, S.D. 0.6 cm; mean weight 1.7 g, S.D. 0.7 g, n = 88) that were used in chapter 3. Mean GSI was 5.7 (S.D. 0.5) and 2.6 (S.D. 0.3) respectively. There was a significant difference in the GSI between these two groups of males (Mann-Whitney *U* Test, $P < 0.001$). The mean GSI of reproductive female sunbleak (mean total length 6.2 cm, S.D. 0.8 cm; mean weight 2.5 g, S.D. 0.5 g, n = 56) was compared with a group of non reproductive sunbleak (mean total length 5.7 cm, S.D. 1.1 cm; mean weight 1.6 g, S.D. 0.2 g, n = 64) used in chapter 3. Mean GSI was 17.2 (S.D. 7.7) and 6.7 (S.D. 3.0) respectively. There was a significant difference in the GSI between these two groups of males (Mann-Whitney *U* Test, $P < 0.001$). These results show that visual identification was an accurate measure of reproductive condition.

5.4. DISCUSSION

The results of this study provide evidence of heterospecific recognition, showing that reproductive chemical cues released by topmouth gudgeon are recognised by reproductive sunbleak. An increase in swimming activity and vagility was only observed in reproductive sunbleak when exposed to water conditioned by reproductive, but not non reproductive topmouth gudgeon. Furthermore, since non reproductive sunbleak did not

respond to any stimulus odour cues it seems that the signal is only relevant to individuals in a reproductive condition.

Olfactory sensitivity to heterospecific chemical cues has been demonstrated in other studies (see Burnard *et al.*, 2008). Notably, the olfactory system of the crucian carp is sensitive to the goldfish sex pheromones 17α,20β-dihydroxy-4-pregnen-3-one and Prostaglandin F2α (Bjerselius & Olsen, 1993). However in this current study, an induced behavioural response suggests that the heterospecific chemical cue is not only recognised but is also a relevant signal (Gerhardt *et al.*, 1994) (i.e. there is a motivation to respond). The results of this current study are preliminary: recognition and motivation to respond to a reproductive heterospecific signal in sunbleak does not imply with certainty that it has a function in the reproductive chemical communication of the species. Interestingly, reproductive male sunbleak also responded to reproductive female topmouth gudgeon conditioned water, despite the apparent lack of response in male topmouth to this odour. The type of response was similar to that observed in male sunbleak during exposure to reproductive female sunbleak conditioned water.

In fishes, it has been suggested that seasonal olfactory sensitivity to reproductive chemical cues occurs due to an increases in the number of crypt cells located at the epithelium surface (Hamdani *et al.*, 2006). Therefore responses by sunbleak to reproductive topmouth gudgeon during the reproductive season could occur because they are more sensitive to the cue released by reproductive topmouth gudgeon than non reproductive sunbleak. However, changes in behaviour (as opposed to EOG responses) imply not only that signal recognition has been achieved, but also that a discriminatory response has been elicited from the receiver. Also, heterospecific responses to cues released by female topmouth gudgeon are sex specific (reproductive male sunbleak respond but reproductive females do not) suggesting an underlying relevance to males.

In laboratory experiments Gozlan *et al.*, (2005) showed that a complete inhibition of spawning occurred in sunbleak when exposed to water conditioned by reproductive topmouth gudgeon. This was attributed to the rosette agent, a parasitic protozoan, of which topmouth gudgeon are a healthy carrier (Gozlan *et al.*, 2005). This current study provides preliminary scope for a new hypothesis regarding the inhibition of sunbleak spawning. Reproductive signals released by topmouth gudgeon are perceived by sunbleak and induce changes in behaviour that imply that the signal contains relevant information. As reproductive chemical cues released by topmouth gudgeon are recognised by sunbleak during the spawning season (chapter 3) it is possible that intraspecific reproductive chemical communication in this species is inhibited by the addition of heterospecific cues derived from topmouth gudgeon. Further research is required to determine if spawning inhibition in sunbleak occurs when exposed to topmouth gudgeon that do not harbour the parasite.

Previous authors have documented responses to conspecific odours. The three spined stickleback recognises and responds to odour from the distantly related guppy *Poecilia reticulate* (Peters) by changing its baseline behaviour (head up and head down postures) (Mclennan, 2003). The pearl danio *Brachydanio albolineatus* (Blyth) is repelled by the odour of reproductive zebra fish *Brachydanio rerio* (Hamilton-Buchanan) (Bloom & Pearlmutter, 1978). This is thought to be a population isolating device in two closely related species that may act to aid speciation (Bloom & Pearlmutter, 1978). This explanation is unlikely in topmouth gudgeon and sunbleak as they are not closely related and further have evolved in geographical isolation.

If responses to heterospecific odours are interpreted as confusion between similar reproductive cues, there are three possible explanations. Firstly, there is a common component to the olfactory cue mixture (Sorensen *et al.*, 1998) that is recognisable across species boundaries. Secondly, the cue is the same in both species (Olsen *et al.*, 2000). Thirdly,

the receiver makes mistakes (Johnson, 1996). As heterospecific responses only occurred in sunbleak (and topmouth gudgeon did not respond) the cue cannot be the same in both species. It is possible that specific components of the cue are the same in both species. For example, an additional component could be present in cues released by topmouth gudgeon that is necessary for responses in this species to occur. The addition of this component however may not affect recognition of the cue in sunbleak. Alternatively, sunbleak could be misinterpreting the signal as a conspecific cue. As the same type of responses (searching behaviour) appear to be initiated in sunbleak in response to heterospecific topmouth gudgeon cues as that observed during exposure to conspecific cues (chapter 3), both explanations provide scope for further study. The bioassay used in this study was designed to determine a response to heterospecific water, the first documented in these respective species. The results present the possibility that two species that have evolved in geographical isolation have coevolved similar reproductive cues. Documenting functional responses to reproductive chemical cues in the respective species (as outlined in chapter 3, section 3.4) would allow bioassays to be developed to discriminate between the two remaining possibilities that either the same or different functions have been evoked in the two species. Here, the hypothesis that sunbleak reproduction is inhibited by cues released by topmouth gudgeon could be further investigated.

CHAPTER 6. ISOLATION OF ACTIVE COMPOUNDS FROM CONDITIONED WATER IN REPRODUCTIVE MALE TOPMOUTH GUDGEON *PSEUDORASBORA PARVA*

6.1. INTRODUCTION

The conserved nature of olfactory systems across different taxa (Hildebrand & Shepherd, 1997; Wyatt, 2003) suggests that identification of the compounds used as reproductive chemical cues in teleosts could provide important information concerning olfactory processes throughout the animal kingdom (Sorensen *et al.*, 1998). As an example, the use of pheromone 'blends' (multi-component pheromones) is considered likely in fishes (Sorensen *et al.*, 1998) which would parallel the olfactory systems of insects (Kaissling, 1996). The pheromone processing apparatus (glomerular processing) also appears similar in fishes and insects (see Sorensen *et al.*, 1998 for a review). The authors suggest that organisation of olfactory apparatus in fishes may also apply to the mammalian vomeronasal system.

The aim of a bioassay guided separation is the isolation of active compounds. Here the compound(s) that elicit responses can be pooled from large amounts of conditioned water. The eluate (concentrate) is separated using high performance liquid chromatography (HPLC) into fractions of signal or retention time windows. These fractions are then tested in behavioural assays to determine those that elicit a response and therefore contain active compounds. Subsequent further analytical separation may allow active compounds to be separated. Bioassay guided separation has been used successfully in the sea lamprey (Li *et al.*, 2002) and the goldfish (Sorensen *et al.*, 2005b).

Isolation of biologically active pheromone compounds from conditioned water has been achieved in many fishes using solid phase extraction (SPE) (Sorensen & Stacey, 2004). As all identified sex pheromones in freshwater fishes (to date-excluding the lamprey) are hormonally derived (Stacey & Sorensen, 2002) and are steroids or prostaglandins, isolation is attained using C-18 cartridges (see chapter 2, section 2.4.3). As, according to the goldfish model, prostaglandins are released by females and steroids by males, C-18 cartridges are particularly useful allowing active compounds from both sexes to be isolated using only one technique. The range of species where sex pheromones have been isolated from conditioned water using C-18 cartridges is great and includes the goldfish (Sorensen *et al.*, 2005b), round goby (Belanger *et al.*, 2004) and Mozambique tilapia *Oreochromis mossambicus* (Peters) (Miranda *et al.*, 2005).

Whilst C-18 cartridges are known to yield components of sex pheromones from fishes, examples are known where active components are not readily isolated using this technique. In the peacock blenny *Salaria pavo* (Risso) (Serrano *et al.*, 2008) and the Eurasian ruffe *Gymnocephalus cernuus*, L, (Sorensen *et al.*, 2004) C-18 cartridges were ineffective in isolating active components of male pheromones. Due to the large range of possible cartridges available for selection, the identification of a viable one could involve extensive research. However, the solvent (holding water) could be removed and the sample (chemical cue) concentrated using a freeze drier providing the active components are not too volatile.

The aim of this investigation is to successfully isolate active components from holding water in reproductive male topmouth gudgeon. On successful isolation, separation by retention time using HPLC will aim to identify the active fractions of a reproductive male released cue from the inactive components. As the use of C-18 cartridges have been found to be an ineffective method of isolating reproductive chemical cues in some teleosts, enrichment using a freeze drier was also tested.

6.2. MATERIALS AND METHODS

This study was conducted using a bioassay guided separation in three steps consisting of sample preparation, separation and detection. Firstly active components (of water conditioned by male topmouth gudgeon) were pooled using two different techniques, solid phase extraction (SPE) and via a freeze drier. These samples were subsequently tested for a behavioural response in female topmouth gudgeon. Secondly, SPE extract and freeze dried extract were fractioned using HPLC and each time fraction tested for a behavioural response in female topmouth gudgeon. Thirdly, active fractions were analysed using Nuclear Magnetic Resonance (^{1}H-NMR).

6.2.1. Conditioning of water

Holding water was conditioned from reproductive and non reproductive males according to the protocol described in chapter 2, section 2.4.2. Gonadal Somatic Index was measured from all fish according to the protocol described in chapter 2, section 2.1.2.

6.2.2. Extraction of active compounds using SPE

Active components were isolated from holding water using SPE according to the protocol described in chapter 2, section 2.4.3. A control blank sample (deionised water) was also prepared. Samples were stored for approximately 2 weeks in a -70 °C freezer prior to use in experiments. This enabled samples to be prepared in advance of behavioural experiments.

6.2.3. Evaporation using a freeze drier

Active components were isolated from holding water using a freeze drier according to the protocol described in chapter 2, section 2.4.4. A control blank sample (deionised water)

was also prepared. Samples were stored for approximately 2 weeks in a -70 °C freezer prior to use in experiments. This enabled samples to be prepared in advance of behavioural experiments.

6.2.4. Experimental design

Experiments were conducted according to the protocol described in chapter 4, section 4.2.1. Twelve individual reproductive females were tested for a response to male derived compounds. GSI was measured from all fish according to the protocol described in chapter 2, section 2.1.2. 1 ml of SPE eluate and freeze dried extract was dissolved in 6 l of dechlorinated water for use in experiments.

6.2.5. Experimental protocol

Experimental procedure was conducted according to chapter 4, section 4.2.2. Swimming activity was found to be an accurate parameter for identifying a response to reproductive male released cues (chapter 4) and was quantified in this investigation. Individual test females were exposed to control water for 45 minutes followed by stimulus water for 45 minutes. On identification of a generic change in swimming activity, headstand courtship responses were quantified during exposure to the active substance.

6.2.6. Water extract fractionation on HPLC

HPLC analysis was performed at Cardiff University. Four samples of conditioned water were prepared for each of the two methods, then extracted and pooled into one sample (20 ml). This sample was subsequently dried in a rotary evaporator and reconstituted in 1 ml of distilled water. An aliquot (25 µl) of the reconstituted sample was loaded onto an ACE 3 AQUA column (Hichrom® 12.5cm*2.1mm i.d) fitted with a phenomenex® security guard

column (C18) and developed using a linear gradient of 0.3% v/v formic acid against ACN 0.3 % formic acid from 0% to 90% over 25 min with an isocratic phase from 25 minutes to 45 minutes at 0.2 ml/min (Thermoseparation P400). Fractions of 5 minutes were collected from 5 minutes to 45 minutes after UV detection at 200 - 600 nm (PDA, UV6000 Thermofinnigan®). The HPLC fractions were labelled according to the time at which they eluted. Twenty HPLC runs were fractionated. All HPLC fractions were dried down in a rotary evaporator and each fraction reconstituted to a final solution in 1 ml of distilled water.

6.2.7. Experimental design

Experiments were conducted according to the design described in chapter 4, section 4.2.2 using 3 test arenas but with the following exception. Due to the preparation and fractionation process it was possible that low concentrations of compounds would be yielded in each fraction. Therefore, to reduce the likelihood that active compounds would be present in concentrations too low to induce a response in recipients, 100 µl of each fraction was pipetted directly into each test arena (25 µm at 10 cm inwards from each side and 50 µl in the centre of each arena). To ensure that the mode of delivery did not impact on the behaviour of the test fish, a separate experiment was undertaken. Here, 100 µl of distilled water was pipetted into each tank (in the same manner as described above) as a stimulus and activity of the recipient compared with that of a preceding control period (with no pipetting).

6.2.8. Experimental protocol

Experimental procedure was conducted according to chapter 4, section 4.2.2 where test fish were exposed to control and stimulus water. Swimming activity was found to be an accurate parameter for identifying a response to reproductive male released cues (chapter 4) and was quantified in this investigation. Each trial lasted 40 minutes with a 20 minute control

period (100 µl of distilled water) and a 20 minute stimulus period. Ten replicate reproductive females were tested for their response to each individual fraction. Each fraction was tested using different females. On identification of a generic change in swimming activity, headstand courtship responses were quantified during exposure to the active fraction. GSI was measured for all fish according to the protocol described in chapter 2, section 2.1.2.

6.2.9. Nuclear Magnetic Resonance (NMR) analysis

The active fraction was dried (GeneVac) and reconstituted in D_2O (Aldrich, 99.9%) for proton-NMR measurements on a Bruker 240 MHz FT-NMR with presaturation.

6.3. RESULTS

6.3.1. Response to concentrated samples

Results show that extraction of reproductive male cues responsible for evoking behavioural responses in female topmouth gudgeon is viable using SPE. There was a significant increase in swimming activity between control and stimulus time periods when reproductive females were exposed to SPE elute derived from reproductive male conditioned water (Wilcoxon signed ranks test n = 12, $P < 0.01$) (Fig. 6.2). There was no significant increase in swimming activity between control and stimulus periods when reproductive females were exposed to SPE elute derived from non reproductive males ($P = 0.51$) or from SPE elute derived from control (non-conditioned) water ($P = 0.96$).

Results show that concentration of reproductive male cues responsible for evoking behavioural responses in female topmouth gudgeon is viable using a freeze drier. There was a significant increase in swimming activity between control and stimulus when reproductive females were exposed to freeze dried extract derived from reproductive male conditioned water (Wilcoxon signed ranks test, n = 12, $P < 0.01$) (Fig. 6.2). There was no significant

increase in swimming activity between control and stimulus periods when reproductive females were exposed to freeze dried extract derived from non reproductive males ($P = 0.73$) or from freeze dried extract derived from control (non-conditioned) water ($P = 0.51$).

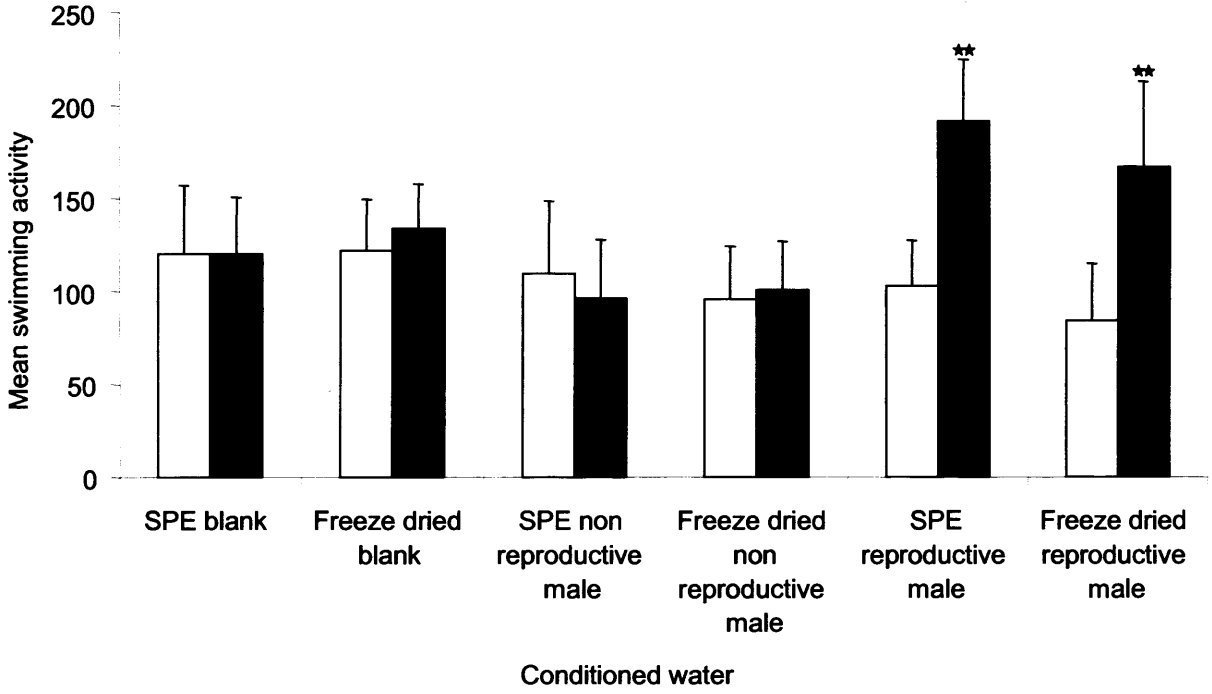

Figure 6.1. The swimming activity of reproductive female topmouth gudgeon *Pseudorasbora parva* in response to a range of odour stimuli extracted from male-conditioned or non-conditioned water. Means ± SE are presented separately for two extraction techniques: SPE = solid phase extraction (n = 12) and Freeze-dried extraction (n = 12). ** represents *P*-values between 0.01 and 0.001. Clear bar = response to control water and solid bars = response to stimulus. All significant responses represent an increase in swimming activity.

6.3.2. Response to retention time fractions

Results show that separation of active reproductive chemical cues was achieved using HPLC. There was a significant increase in swimming activity when reproductive females were exposed to time fraction 31-35 derived from SPE elute originating from reproductive male conditioned water (Wilcoxon signed ranks test (n = 10, $P < 0.01$) (Fig.6.3). There was no significant increase in swimming activity between control and stimulus when reproductive females were exposed to all other time fractions (n = 10, fraction 0-5 $P = 0.58$,

fraction 6-10 P = 0.99, fraction 11-15 P = 0.58 fraction 16-20 P = 0.51, fraction 21-25 P = 0.11, fraction 26-30 P = 0.73, fraction 36-40 P = 0.38 and fraction 41-45 P = 0.65). There was no significant increase in swimming activity between control and stimulus when reproductive females were exposed to distilled water pipetted into the test arenas as a stimulus (Wilcoxon signed ranks test (n = 10, P = 0.92).

The findings were replicated for freeze-dried samples. There was a significant increase in swimming activity between control and stimulus when reproductive females were exposed to time fraction 31-35 freeze dried extract originating from reproductive male conditioned water (Wilcoxon signed ranks test n = 10, $P < 0.01$) (Fig. 6.4). There was no significant increase in swimming activity between control and stimulus when reproductive females were exposed to all other time fractions (n = 10, fraction 0-5 P = 0.65, fraction 6-10 P = 0.80, fraction 11-15 P = 0.76, fraction 16-20 P = 0.96, fraction 21-25 P = 0.88, fraction 26-30 P = 0.20, fraction 36-40 P = 0.88 and fraction 41-45 P = 0.88).

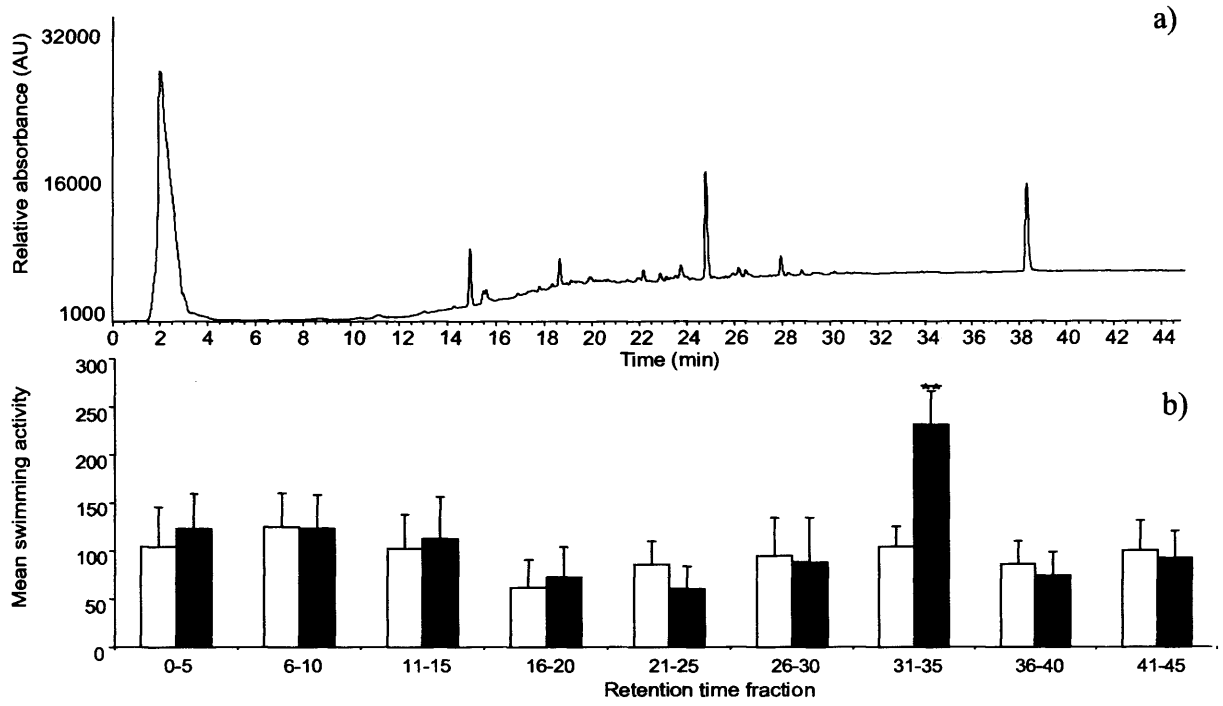

Figure 6.2. a) High performance liquid chromatography (HPLC) chromatogram of solid phase extraction (SPE) elute derived from reproductive male topmouth gudgeon *Pseudorasbora parva* conditioned water **b)** corresponding time fraction responses of reproductive females (n = 10). ** represents *P*-values between 0.01 and 0.001. All significant responses represent an increase in swimming activity.

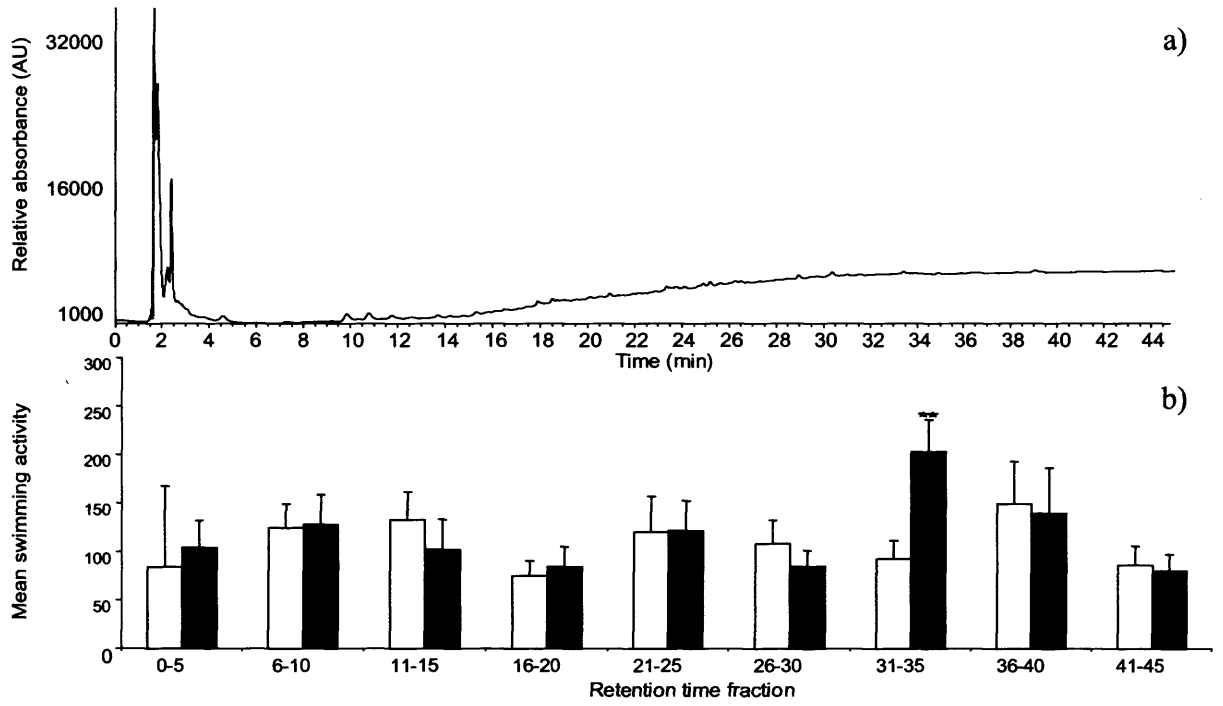

Figure 6.3. a) High performance liquid chromatography (HPLC) chromatogram of freeze dried extract derived from reproductive male topmouth gudgeon *Pseudorasbora parva* conditioned water **b)** corresponding time fraction responses of reproductive females (n = 10). ** represents *P*-values between 0.01 and 0.001. All significant responses represent an increase in swimming activity.

6.3.3. Induction of courtship responses during exposure to SPE eluate and fraction 31 -35 derived from reproductive males

42% and 33% of reproductive females performed headstands in response to SPE extract (n = 12) and fraction 31-35 (n = 12) derived from reproductive males. No headstands were performed in control periods. There was a significant increase in swimming activity between control periods and exposure both to SPE extract ($P = 0.003$) and fraction 31-35 ($P = 0.008$) (Wilcoxon signed ranks test n =12).

Mean GSI of reproductive males (mean total length 5.5 cm, S.D. 1.2 cm; mean weight 5.9 g, S.D. 1.3 g, n = 20) and non reproductive males (mean total length 6.6 cm, S.D. 0.9 cm; mean weight 6.2 g, S.D. 1.0 g, n = 20) used to condition water in this study was 2.6 and 1.0 respectively. There was a significant difference in GSI between these two groups (Mann-Whitney *U* Test, $P < 0.001$). Reproductive females (mean total length 5.1 cm, S.D. 1.5 cm; mean weight 1.5 g, S.D. 0.6 g, n = 276) used in this study were compared with a group of non reproductive females gudgeon (mean total length 5.6 cm, S.D. 1.1 cm; mean weight 1.6 g, S.D 0.2 g, n = 24) used as test fish in chapter 3. GSI of these two groups was 6.7 (SD 3.0) and 14.9 (SD 7.8) respectively. There was a significant difference in GSI between these two groups (Mann- Whitney *U* Test, $P < 0.001$). These results show that visual identification was an accurate measure of reproductive condition.

6.3.4. Nuclear Magnetic Resonance (NMR) analysis

The NMR scan revealed that compounds are present in fraction 31-35 in low yields (Fig. 6.5) but did not contain enough substance to allow identification of the compound or to attempt subsequent carbon NMR and 2 dimensional correlation spectroscopy e.g. Homonuclear Correlation Spectroscopy (COSY) or Heteronuclear Single Quantum Coherence (HSQC). The few visible peaks indicate aliphatic protons: methylene protons (CH_2) around 2ppm and methin (CH) around 3ppm. The latter is likely to carry a polar group, e.g. hydroxy (OH). These findings do not contradict the observation of behaviour in separation and detection i.e. lipophilic and not UV-active. The NMR readings have to be treated cautiously, as it is not certain that they actually belong to the compound responsible for the behavioural response.

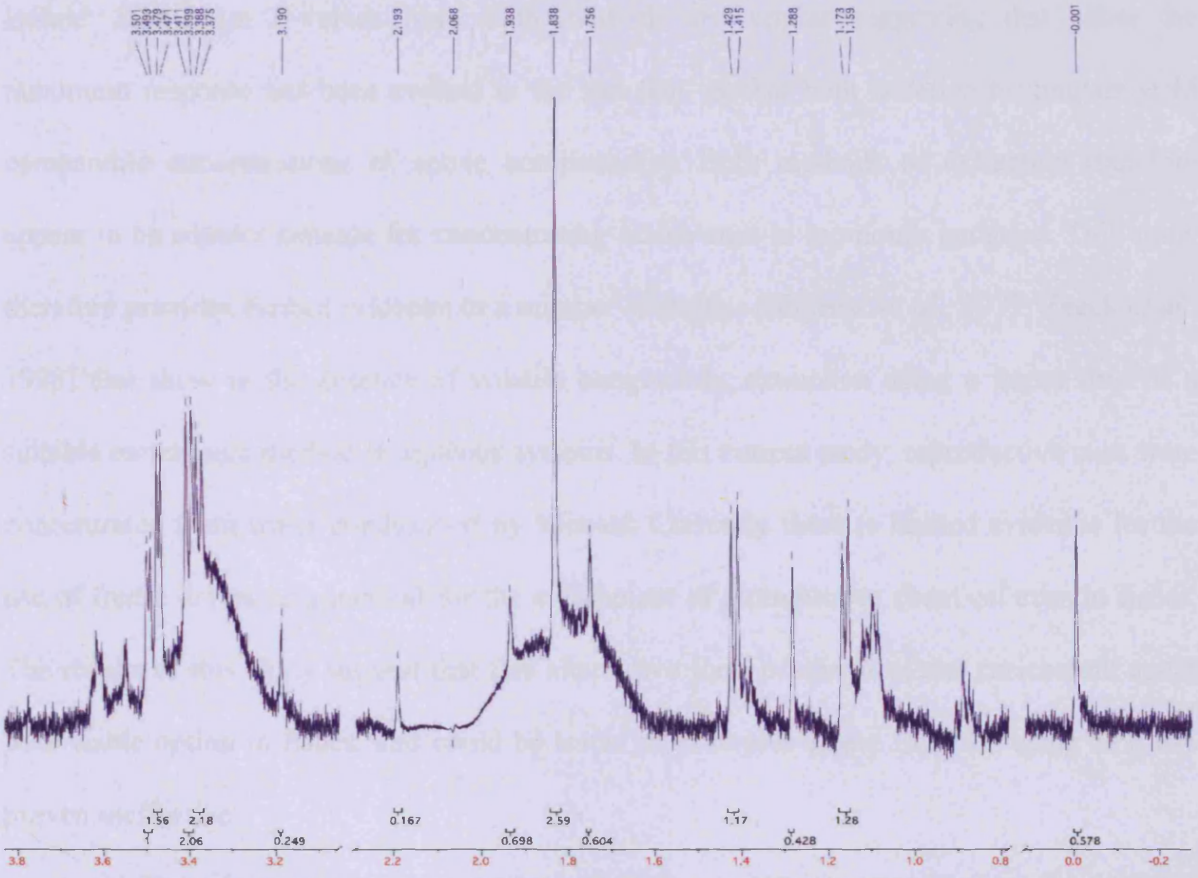

Figure 6.4. Nuclear magnetic resonance (Proton-NMR) spectrum of fraction 31-35 extracted using solid phase extraction (SPE) and derived from reproductive male topmouth gudgeon *Pseudorasbora parva* conditioned water.

6.4. DISCUSSION

The results of this study show that reproductive male cues can be successfully isolated from conditioned water. SPE elute and freeze dried extract derived from reproductive male conditioned water both evoked strong behavioural responses in reproductive females. The results of this investigation are in agreement with other studies using species that exhibit male nest guarding, notably that of the sea lamprey (Li *et al.*, 2002) and the round goby (Belanger *et al.*, 2004). Here, reproductive male conditioned water isolated using C-18 SPE cartridges were shown to evoke behavioural responses in reproductive females (Li *et al.*, 2002; Belanger *et al.*, 2004).

In this study, freeze dried extract derived from reproductive males evoked behavioural responses in reproductive females to a similar degree as that evoked using SPE isolute. Significant *P*-values from both methods are similar suggesting that either the maximum response has been evoked in the test fish, or that both isolation techniques yield comparable concentrations of active compound(s). Both methods of extraction therefore appear to be equally suitable for concentrating active cues in topmouth gudgeon. This study therefore provides further evidence to a number of studies (Mcleese *et al.*, 1977; Zeeck *et al.*, 1998) that show in the absence of volatile compounds, extraction using a freeze drier is a suitable enrichment method in aqueous systems. In this current study, reproductive cues were concentrated from water conditioned by teleosts. Currently there is limited evidence for the use of freeze dryers as a method for the enrichment of reproductive chemical cues in fishes. The results of this study suggest that this alternative form of chemical cue enrichment could be a viable option in fishes, and could be tested in examples where isolation using SPE has proven ineffective.

Behaviour experiments determined that one HPLC time fraction (31-35 min) evoked behavioural responses in reproductive females. No other fraction evoked changes in

swimming activity in either SPE elute or freeze dried extract. This shows that the compound(s) responsible for the behavioural responses in reproductive female topmouth gudgeon in response to reproductive male conditioned water (chapter 3), SPE extract and freeze dried extract are only present in this fraction. These findings are in accordance with similar studies (Li *et al.*, 2002; Belanger *et al.*, 2004) that have identified female responses to specific HPLC time fractions derived from male conditioned water. In particular, Li *et al.*, (2002) identified a response to a retention time fraction that yielded a chromatogram peak. Subsequent analysis enabled the precise compound (7α, 12α, 24-trihydroxy-5α-cholan-3-one 24-sulphate) to be isolated and identified. As the current study did not yield a significant peak on the chromatogram such a direct approach could not be used. However, a further bioassay guided assay focussed on the active fraction could allow the precise retention time of the compound to be elucidated. Pooling the compound would then enable comprehensive analysis using one and two-dimensional NMR methods. This study shows that headstand courting responses are induced by SPE extract derived from male conditioned water. Furthermore, headstand behaviour was induced by active fraction (31-35) separated by HPLC. An increase in swimming behaviour by females that performed headstands in response to male conditioned water (chapter 3), SPE extract and fraction 31-35 suggests that changes in swimming activity and headstand postures are linked.

The chemical structure of the compound(s) responsible for the evoked behavioural response in reproductive females could not be elucidated; however it is possible to deduce some structural characteristics. Firstly, the compound(s) were extracted using C-18 cartridges which retain lipophilic compounds. Secondly, the large retention time is consistent with lipophilic substances. Thirdly, the compounds did not yield a signal in the UV detection and are, therefore, not UV-active, which suggest that they are unlikely to contain carboxyl groups or aromatic systems. Fourthly, the few signals in the NMR indicated aliphatic protons only.

All findings together point to a purely aliphatic and not overly polar compounds and would match the properties of steroids in general.

The results from this study show that active compounds derived from reproductive male conditioned water responsible for inducing an increase in swimming activity in reproductive females can be successfully isolated from conditioned water and separated by retention time using HPLC, factors essential for possible identification in further analytical study.

CHAPTER 7. ELECTRO-PHYSIOLOGICAL RESPONSES TO WATER BORNE ODOURS IN TOPMOUTH GUDGEON *PSEUDORASBORA PARVA*

7.1. INTRODUCTION

Behavioural investigations are commonly used to characterise sex pheromone systems as the identification of induced responses are needed to determine the specific function of the chemical cue (Cole & Smith, 1992; Li *et al.*, 2002; Corkum *et al.*, 2006). However, electrophysiological evidence is an important validation tool, as odours responsible for behavioural responses will be expected to yield olfactometric responses, specifically the recording of an electro-olfactogram (EOG) (see chapter 1 for further details). EOG responses can also be used to provide a basic understanding of how conserved, or conversely how diverse chemical communication systems are between species (Essington & Sorensen, 1996). Known sex pheromones of one species for example, can be tested on other species to determine whether they are perceived. The presence and magnitude of the response can be used as an indication of olfactory divergence between species (Bjerselius & Olsen, 1993).

The electro–olfactogram records a negative electrical potential generated from the olfactory sensory neurons (OSNs) (Ottoson, 1956). According to current understanding, odorants bind to olfactory receptor proteins (ORs) that populate the surface of the OSNs (Ottoson, 1956). These ORs are coupled to G-protein complex which via secondary messengers open Na^+ channels resulting in depolarisation. The measured action potential (EOG) is a separate event, dependent on depolarisation activating voltage gate channels (see Scott & Scott-Johnson, 2002 for a review). Whilst it is generally accepted that the EOG response represents the summated generator potential in the ORNs (Ottoson, 1956) some

authors suggest that inhibitory events may contribute to the EOG. Presently there is limited evidence promoting this view (Scott & Scott-Johnson, 2002), therefore EOG recordings are considered as an accurate measurement of olfactory perception.

EOG recordings are frequently used in combination with behavioural research to provide a comprehensive understanding of olfactory communication (Almeida *et al.*, 2005, Miranda *et al.*, 2005 and see a review by Burnard *et al.*, 2008). In the round goby, recognition of reproductive male derived odour in reproductive females was determined by the magnitude of EOG response to reproductive and non reproductive male odour (Belanger *et al.*, 2004). Larger magnitude EOG recordings were documented in response to reproductive males. Behavioural investigations determined that reproductive females are attracted to reproductive male derived odours that in conjugation with the electrophysiological research provided conclusive evidence of an operating sex pheromone system (Belanger *et al.*, 2004).

The aim of this investigation was to assess olfactory sensitivity in topmouth gudgeon to conspecific odours. EOG recordings in response to reproductive and non reproductive male and female odour were conducted on reproductive males and reproductive females. As previous work by this author has shown that behavioural responses occur in reproductive females in response to reproductive male conditioned water (chapter 3) and males guard nests that females lay eggs into (Maekawa *et al.*, 1996), it was hypothesised that there would be an EOG response in reproductive females to reproductive male odours. In addition, EOG responses to a range of compounds includes an amino acid (L-arginine), bile acid (5α-cyprinol sulphate), hormones (estradiol, testosterone, 11-keto testosterone, testosterone-sulphate and estrone-3 sulphate,) and sex pheromones (17,20β-progesterone, 17,20β-progesterone-sulphated, 17,20β-progesterone-glucuronide and androstenedione) in fishes will provide an insight of olfactory sensitivity in topmouth gudgeon.

7.2. MATERIALS AND METHODS

7.2.1. Conditioning of water and Solid Phase Extraction (SPE) of active compounds

Water was conditioned according to chapter 2, section 2.4.2. Water was conditioned by non reproductive males and females and reproductive males and females. SPE extraction of compounds from conditioned water and a control of dechlorinated tap water were conducted using protocol described in chapter 2, section 2.4.3. GSI was calculated according to chapter 2, section 2.1.2. Elutes were stored at -70 °C for approximately 7 months prior to EOG recordings. Samples were transported to Faro University (Portugal) and stored at -20 °C. Transportation time did not exceed 8 hours.

7.2.2. Fish transportation and holding facilities

Fish (approximately 100 individuals of mixed sex) were transported via the freight company GAC Logistics from Bournemouth University (UK) to Faro University (Portugal). Study was conducted with the expertise of P. C. Hubbard. At Faro University, fish were held separately outside in 1000 l tanks (i.e. under natural temperature and photoperiod) with filtration and aeration. Fish were fed once a day with Tetrapond 'PondSticks' and allowed to adapt for two weeks before experiments.

7.2.3. Sample preparation

Tenfold dilutions (10^{-3} to $10^{-6)}$ were made from stock solutions of SPE blank, reproductive male, reproductive female, non reproductive male and non reproductive female conditioned water. Dilutions were made using dechlorinated tap water. A 'standard' of 10^{-5} M L-serine was also prepared so recordings of responses to conditioned water could be

normalised for quantitative analysis. L-arginine (10^{-8} M to 10^{-4} M) and 5α-cyprinol sulphate at concentrations ranging from 10^{-9} M to 10^{-5} M were also prepared to access olfactory sensitivity to differing concentrations. Samples of estradiol, testosterone, 11-keto testosterone, testosterone-sulphate, androstenedione, 17,20β-progesterone, 17,20β-progesterone- sulphated, 17,20β-progesterone-glucuronide and estrone-3 sulphate were prepared at 10^{-6} M.

7.2.4. Recordings of the Electro-olfactogram

EOGs were recorded using a protocol based on Hubbard *et al.*, (2002). See Fig. 7.1 for a picture of the electro-olfactory gram rig used for experiments. Topmouth gudgeon were anaesthetised by immersion in water containing 100mg l^{-1} of 3-aminobenzoic acid ethyl ester (MS222) and immobilised with an intramuscular injection of gallamine triethiodide (3mg kg^{-1}) in 0.9% saline. The fish was then clamped in a padded Perspex@ stand wrapped in a wet towel and the eyes covered. The gills were irrigated with dechlorinated, aerated tap water containing MS222 (50 mg l^{-1}) via a plastic tube inserted into the mouth. The flap covering the nostril was cut away exposing the olfactory rosette (Fig. 7.2a). The recording electrode was placed between two adjacent lamellae close to, but not touching, the olfactory epithelium (Fig. 7.2b). The reference electrode was placed lightly on the skin of the head near the nostril and connected to earth via the headstage of the amplifier. The olfactory epithelium was continually irrigated with dechlorinated, charcoal–filtered tap water at a rate of 6 ml min^{-1}. Stimuli were introduced into this flow via a three–way valve for 4 or 5 s.

The voltage signal was amplified using a Grass AC/DC strain gauge (CP122; Astro-Med, West Warwick, RI) with low-pass filter set at 30 Hz. The signal was then digitised (DigiData 1322A, Molecular Devices Corporation, Sunny Vale, CA, USA) and recorded by a PC running Axoscope software (version 9.2, Molecular Devices Corporation). Recording and

reference electrodes were made from borosilicate glass micropipettes filled with 1.0 M NaCl /1% agar (tip diameter 50 – 80µm) connected to Ag/AgCl salt bridge via 3 M KCl.

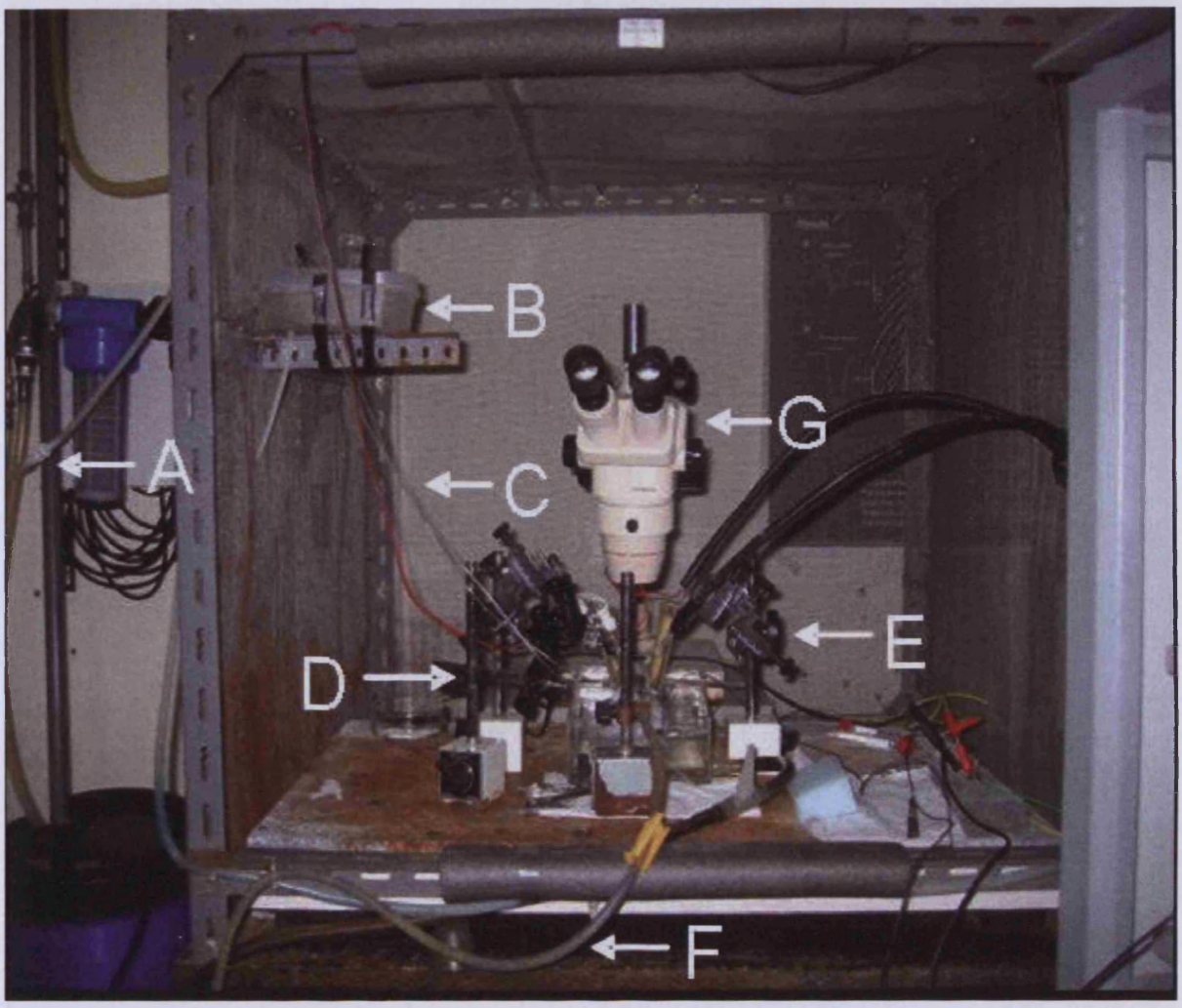

Figure 7.1. Electro-olfactogram (EOG) rig used for experiments. A = input water supply, B = control/stimulus input, C = delivery tube, D = recording electrode, E = reference electrode, F = gill irrigation tube, G = viewing microscope.

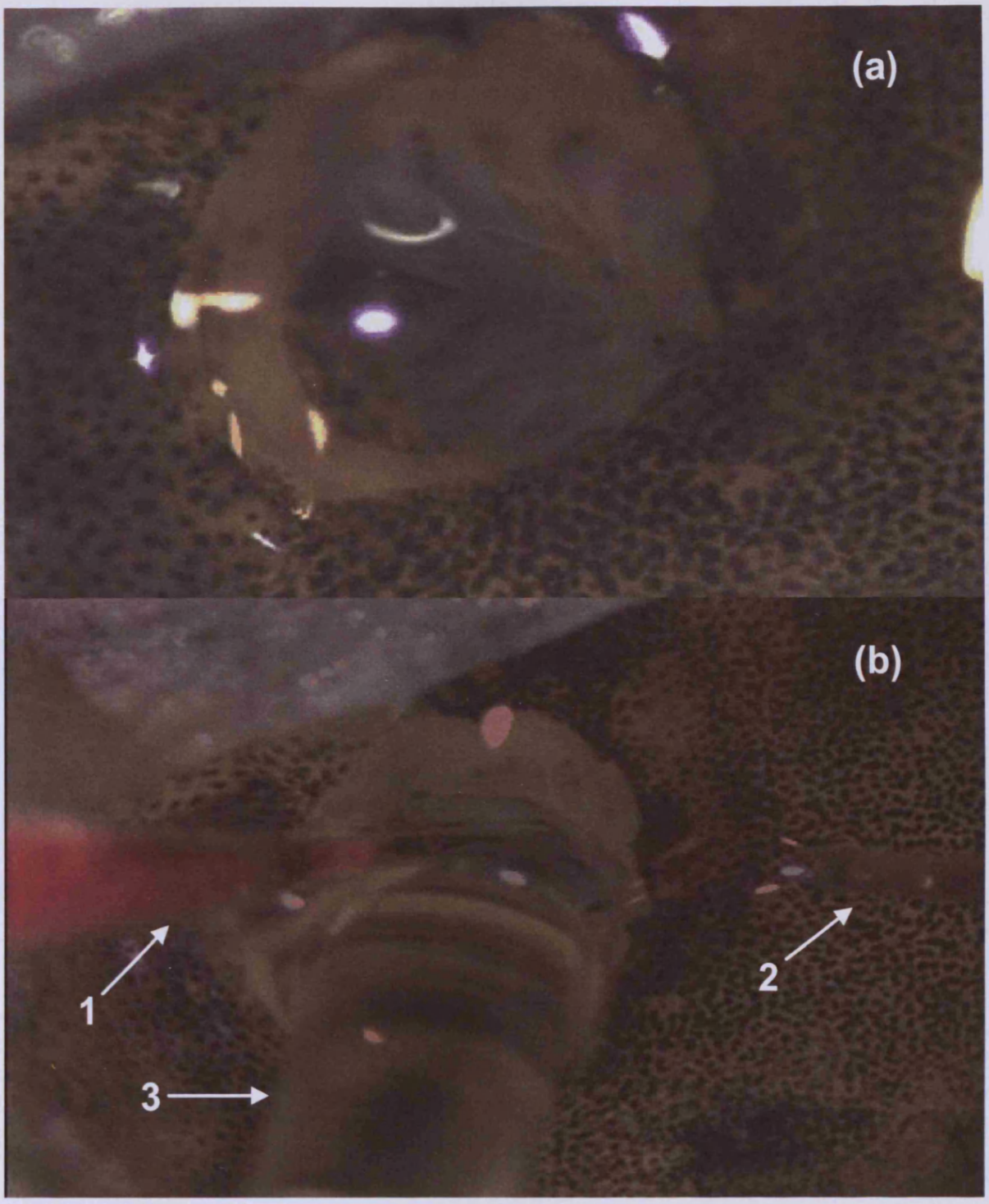

Figure 7.2. a) Exposed topmouth gudgeon *Pseudorasbora parva* olfactory rosette **b)** position of recording electrode (1), reference electrode (2) and delivery apparatus (3).

7.2.5. Experimental design

After the fish and recording electrodes were set in place, the optimal position for the recording electrode was determined using 10^{-5} M L-serine as stimulus (determined by the largest response). To account for the variation of EOG amplitude due to differing olfactory sensitivities between individual fish, all responses were normalised to the previous standard response to 10^{-5} M L-serine run before each treatment group. A control (background water treated in the same way as stimulus water but without the addition of stimulus) was also carried out before each treatment group to eliminate the olfactory response to the water in which the stimulus was prepared. The order in which treatment groups (control, SPE control, non reproductive males and females and reproductive males and females) were tested was randomised. Stimuli were applied in ascending concentration (10^{-6}, 10^{-5}, 10^{-4} then 10^{-3}) to counteract accommodation (Hubbard *et al.*, 2002). This was also minimised by flushing the valve and stimulus lines and allowing at least 1 min between subsequent stimuli. Response to each stimulus was recorded from 6 fish (6 replications). GSI was recorded from all test fish according to chapter 2, section 2.1.2.

6.2.6. Data analysis

The peak amplitude of the EOG test solutions was measured in millivolts. Data were normalised to the EOG response to a 10^{-5} M L-serine solution run prior to each treatment group and blank-subtracted (to the blank run prior to each treatment group). Statistical analyses were performed on normalised data using a Kruskal-Wallis test and a post hoc Mann-Whitney *U* Test.

7.3. RESULTS

The olfactory system of topmouth gudgeon proved to be sensitive to both reproductive male and female odours giving large amplitude EOG responses typical of fishes; a rapid negative deflection upon the arrival of the stimulus at the olfactory epithelium followed by a period of adaptation and a return to baseline after the stimulus was removed (Fig.7.3).

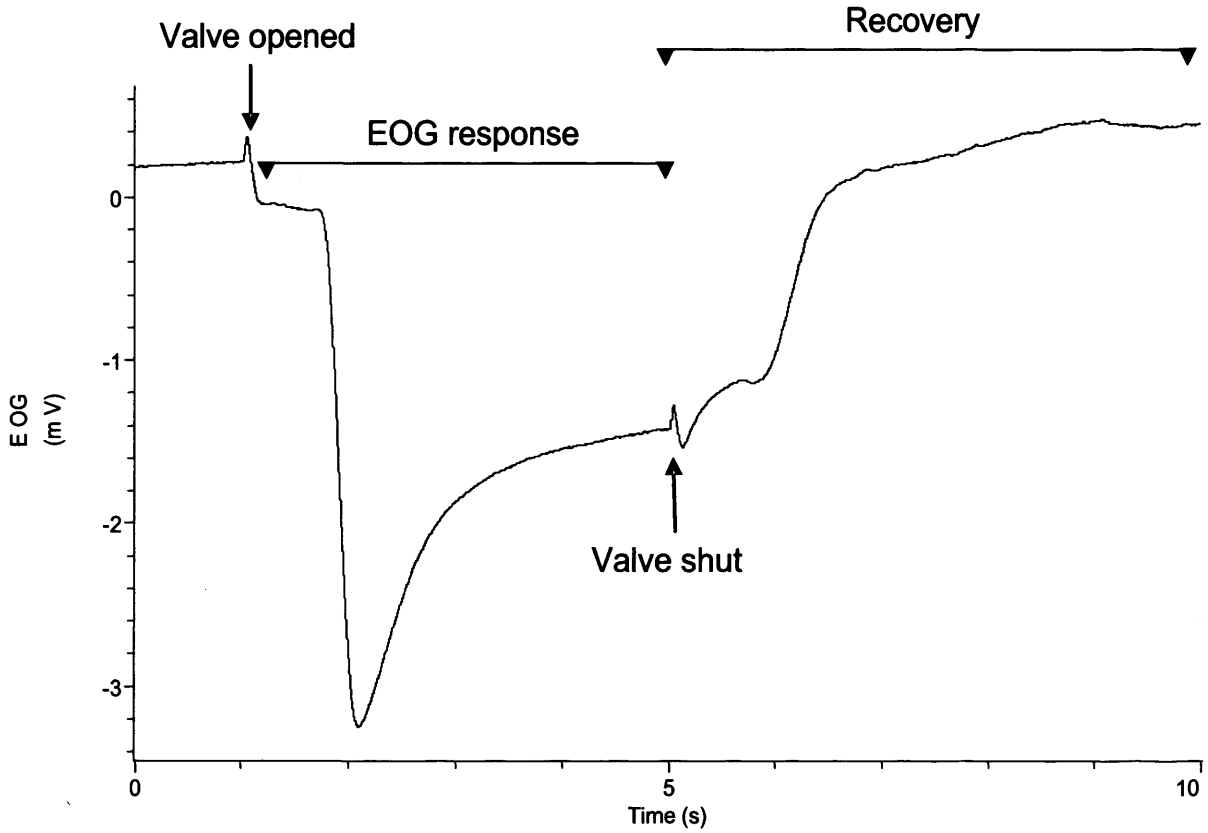

Figure 7.3. Electro-olfactogram (EOG) trace from reproductive female topmouth gudgeon *Pseudorasbora parva* during exposure to reproductive male topmouth gudgeon conditioned water showing valve opened to allow exposure of the olfactory epithelium to stimulus, the resulting EOG response and the valve shut to stop exposure and the subsequent recovery. Change in EOG when valve opens and shuts is most likely due to flow rate fluctuation over the olfactory epithelium.

In reproductive females there were significant differences in the amplitude of responses to conspecific water (Kruskal-Wallis test $P < 0.01$, n = 24) at dilution 10^{-3} (Fig.

7.5a; 7.5b). There was a significant difference between responses when reproductive females were exposed to reproductive and non reproductive male odours (Mann-Whitney *U* Test, $P < 0.01$, n = 6) and reproductive and non reproductive female odours at dilution 10^{-3} (Mann-Whitney *U* Test, $P < 0.01$, n = 6). Mean female EOG response to SPE blank was extremely low at 0.42 mV (S.E = 0.09).

In reproductive males there were significant differences in the amplitude of responses to conspecific water (Kruskal-Wallis test $P < 0.001$, n = 24) at dilution 10^{-3} (Fig. 7.5a; 7.5b). There were significant differences between responses when reproductive males were exposed to reproductive and non reproductive male odours (Mann-Whitney *U* Test, $P < 0.01$, n = 6) and reproductive and non reproductive female odours (Mann-Whitney *U* Test, $P < 0.01$, n = 6) at dilution 10^{-3}. Mean male EOG response to SPE blank was low at 0.7 mV (S.E = 0.22).

The normalised EOG amplitude of responses to the amino acid L- arginine and the bile acid 5α-cyprinol sulphate showed a clear concentration dependence (Fig. 7.5) with no evidence of reaching a plateau at the highest concentration tested (10^{-4} M and 10^{-5} M respectively) and thresholds of detection of approximately 10^{-8} M. There were significant differences in the amplitude of response to 5α-cyprinol sulphate at concentration 10^{-5} in males (Kruskal-Wallis test $P < 0.01$, n = 6; post hoc Mann Whitney *U* test $P < 0.05$, n = 6) and females (Kruskal-Wallis test $P < 0.01$, n = 6; post hoc Mann Whitney *U* test $P < 0.05$, n = 6). There were significant differences in the amplitude of response to L-arginine at concentration 10^{-4} in females (Kruskal-Wallis test $P < 0.01$, n = 6; post hoc Mann Whitney *U* test $P < 0.05$, n = 6) (Fig. 6.6a, 6.6b). Male topmouth gudgeon showed a large magnitude response to 17-20 BP (free, conjugated and sulphated forms) and 11-keto testosterone (Fig. 7.7).

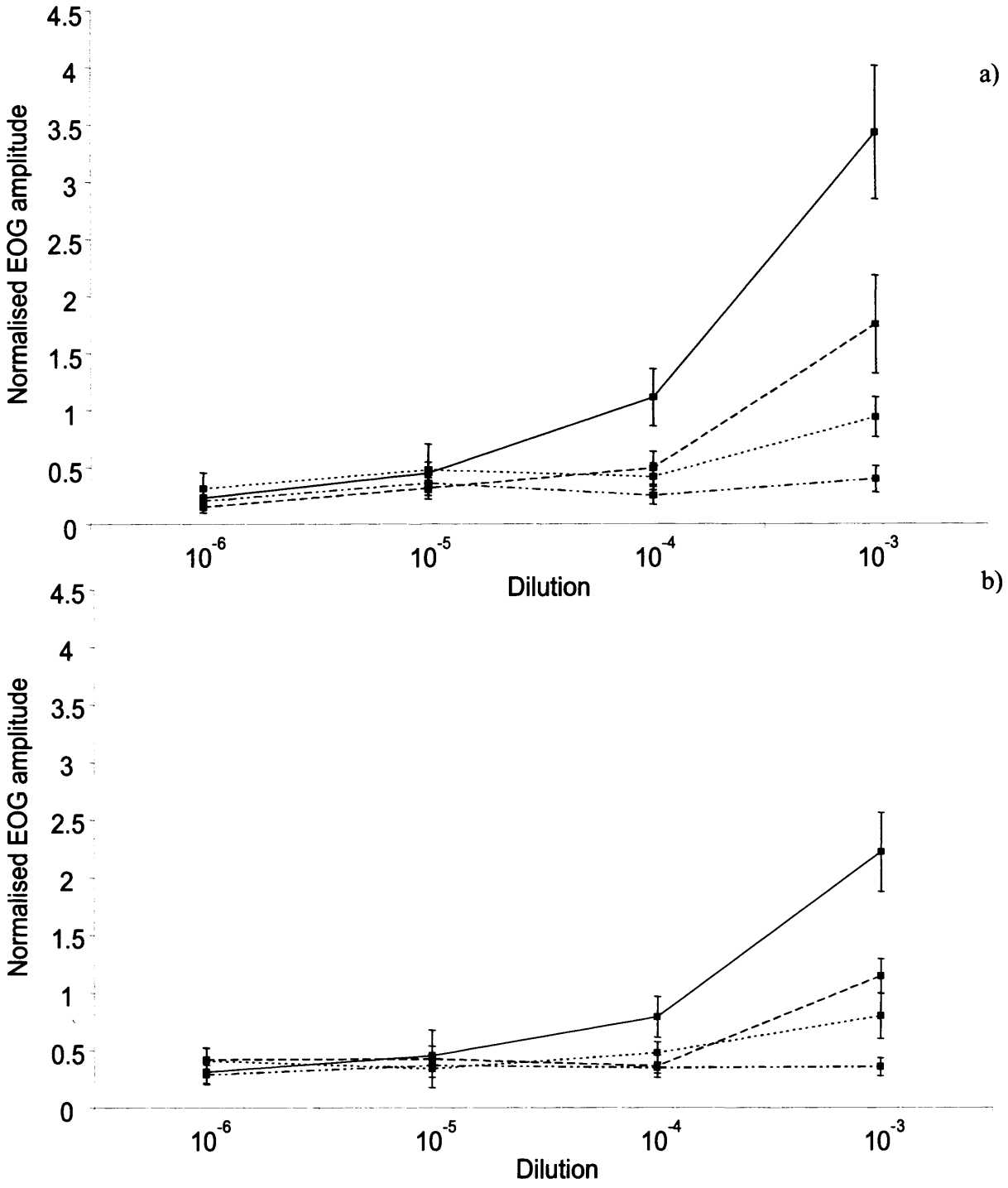

Figure 7.4. Mean olfactory response of topmouth gudgeon *Pseudorasbora parva* to conditioned water. **a)** Mean response of reproductive males (n = 6) to conditioned water **b)** Mean response of reproductive females (n = 6) to conditioned water. (—) = response to reproductive male, (– –) = response to reproductive female, (·····) = response to non reproductive male and (- - -) = response to non reproductive females. Data are shown as mean ± S. E. and are blank corrected and normalised to the amplitude of response to 10^{-5} M L-serine.

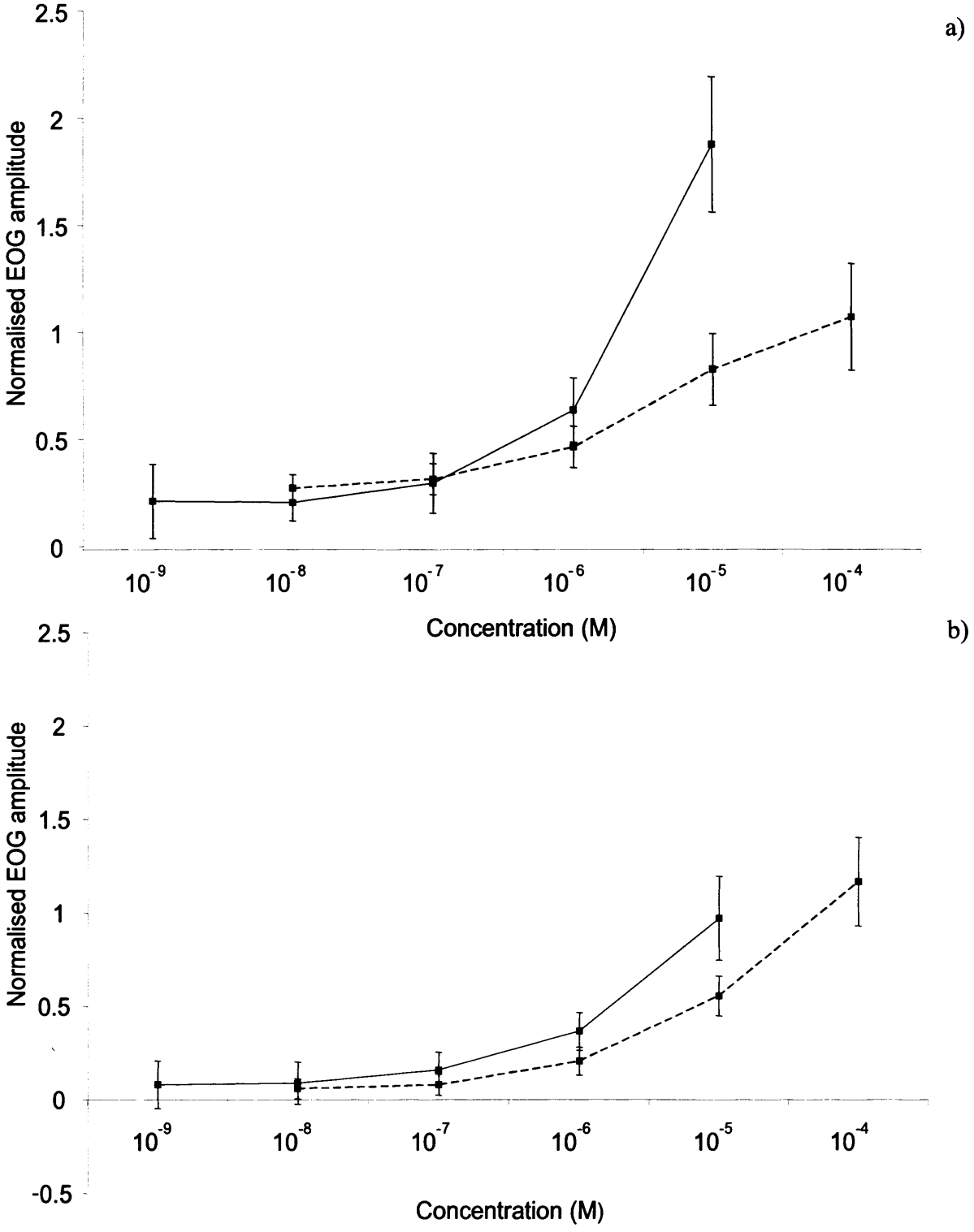

Figure 7.5. Mean olfactory response of topmouth gudgeon *Pseudorasbora parva* to 5α-cyprinol sulphate and L-arginine. **a)** Mean response of reproductive males (n = 6) to 5α-cyprinol sulphate (—) and L-arginine (- - -). **b)** Mean response of reproductive females (n = 6) to 5α-cyprinol sulphate (—) and L-arginine (- - -). Data are shown as mean ± S. E. and are blank corrected and normalised to the amplitude of response to 10^{-5} M L-serine.

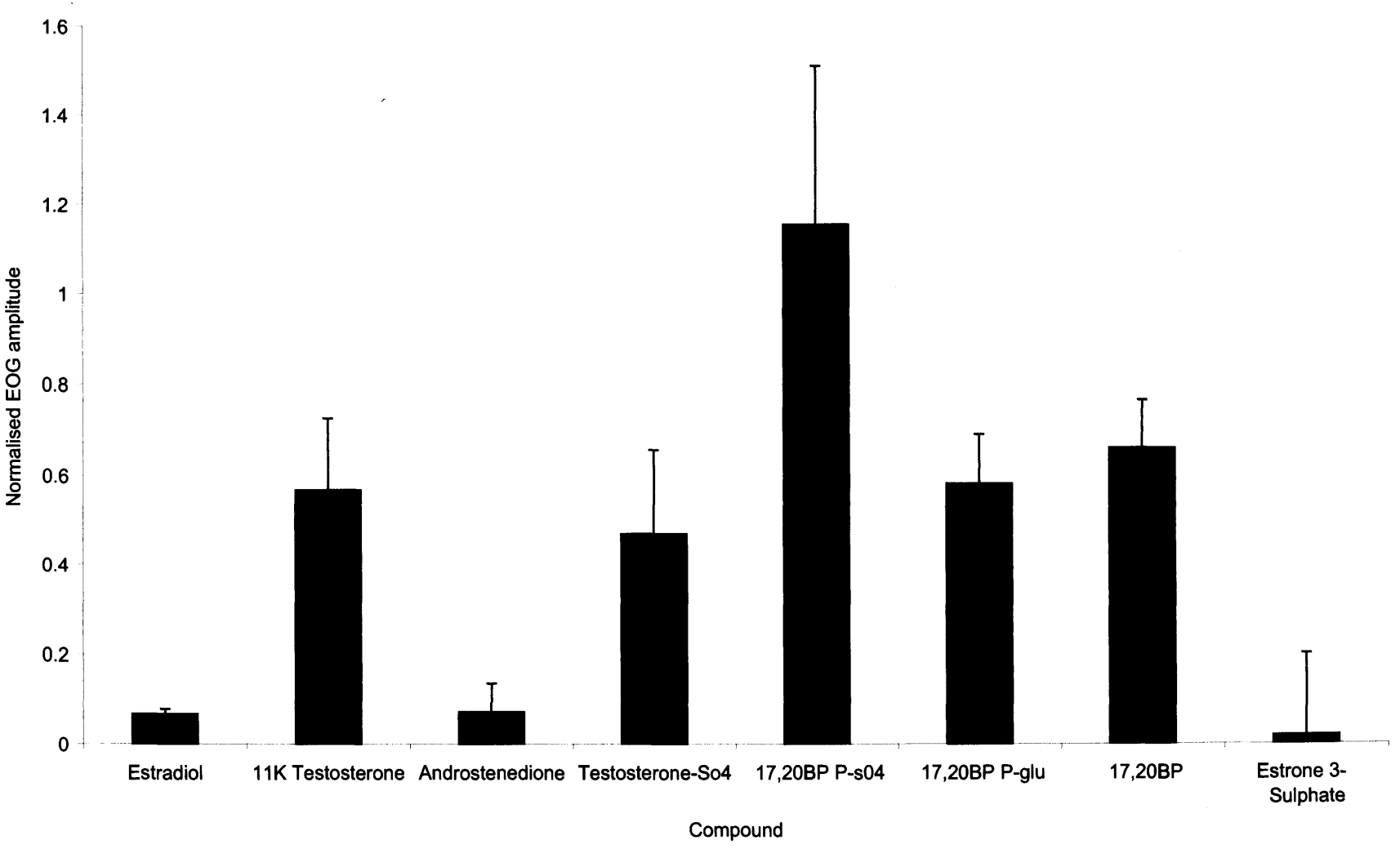

Figure 7.6. Mean electro-olfactogram (EOG) response of reproductive male topmouth gudgeon *Pseudorasbora parva* (n = 6) to selected compounds (10^{-6} M). Data are shown as mean ± S. E. and are blank corrected and normalised to the amplitude of response to 10^{-5} M L-serine.

Mean GSI of reproductive males (mean total length 7 cm, SD 1.1 cm; mean weight 6.5 g, S.D. 1.0 g, n = 24) was compared with a group of non reproductive male topmouth gudgeon that were used as test fish for chapter 3 (mean total length 7.3 cm, S.D. 0.8 cm; mean weight 6.0 g, S.D. 1.2 g, n = 40). Mean GSI was 2.7 (S.D. 1.1) and 1.1 (S.D. 0.5) respectively. There was a significant difference in the GSI between these two groups of males (Mann-Whitney *U Test*, $P < 0.001$, n = 54). Mean GSI of reproductive females (mean total length 5.3 cm, S.D. 0.8 cm; mean weight 1.6 g, S.D. 0.6 g, n = 24) was compared with a group of non reproductive female topmouth gudgeon that were used as test fish for chapter 3 (mean total length 7.3 cm, S.D. 0.8 cm; mean weight 6.0 g, S.D. 1.2 g, n = 40). Mean GSI was 2.7 (S.D. 1.1) and 1.1 (S.D. 0.5) respectively. There was a significant difference in the GSI between these two groups of females (Mann-Whitney *U* Test, $P < 0.001$, n = 54). These results show that visual identification was an accurate measure of reproductive condition.

7.4. DISCUSSION

The observed increased olfactory activity in reproductive females following stimulation with reproductive male odour corroborates results from behavioural experiments (chapters 3 and 4) where an increase in swimming activity occurred in reproductive females in response to reproductive male conditioned water. The large magnitude of responses to male released odour imply strong olfactory sensory neuron generator potentials (Ottoson 1956; Getchell, 1974). The increased olfactory receptor cell activity may stimulate a sequence of neuronal events that lead reproductive females to locomotive responses (chapter 3). Responses occur to SPE extract corroborating behavioural results from chapter 5. Also, EOG sensitivity to odours occurred after long term storage, suggesting that active components do not degrade over time when frozen. This would aid subsequent research, particularly chemical analysis which requires an abundance of the active compound.

In addition to olfactory sensitivity to reproductive male odour, an EOG response of large magnitude occurs to reproductive female conditioned water. It is therefore possible that cues released by reproductive female may have a role in the sex pheromone system of topmouth gudgeon, despite the lack of locomotor activity in males when exposed to reproductive female conditioned water (chapter 3). Furthermore, male olfactory sensitivity to 17, 20 BP, (free and conjugated) a female released sex pheromone in a number of fishes (see Burnard *et al.*, 2008 for a review) is large. However the olfactory epithelium is used for the sensory recognition of a great range of odours (Wyatt, 2003) and not just an apparatus to steer spawning behaviour therefore perception of the odour does not equate to its use in reproductive communication.

The identification of an EOG response to reproductive female odours suggests a chemical role for female cues in this species. However no behavioural effect was identified (chapter 3). It is possible that the response of the male to female odour is dependent on the status of the male and the absence of a nest in the bioassay could therefore be responsible for the lack of response. A bioassay that tests the response of reproductive males that are actively guarding a nest (and therefore seeking receptive females) would determine if males do respond to female cues. The results of this current investigation are in accordance with that obtained from study with the round goby. Here, behavioural responses were absent (Marentette *et al.*, 2008) despite the detection of conspecific odours shown by an increase in gill ventilation (Murphy *et al.*, 2001; Belanger *at al.*, 2006). It is possible that in both topmouth gudgeon and the round goby primer responses to female odour may not be coupled with behavioural responses.

A range of hormones (estradiol, testosterone, testosterone-sulphate and estrone-3 sulphate) and a known sex pheromone (androstenedione) elicited little or no EOG response in topmouth gudgeon, effectively ruling them out as candidate pheromones. Even though

perception does not equate to function and is it not possible to make assumptions of the possible identity of sex pheromones as a result of high olfactory sensitivity, it can be used to identify possible candidates that could be tested for a behavioural response in functional bioassays or in primer investigations. In goldfish 17, 20 BP is released by females to elicit both sexual behaviour (Sorensen *et al.*, 1990) and male priming (Dulka *et al.*, 1987) in males. It is possible that 17, 20 BP acts a priming pheromone in male topmouth gudgeon. The largest magnitude EOG response occurs to 17, 20 BP-S04, perhaps suggesting a possible role in the sex pheromone system of topmouth gudgeon. However, as previously described (chapter 3) the accurate measurement of sperm volume and quality is currently challenging in this species, so further study would be difficult. High olfactory sensitivity to 11-keto testosterone suggests that this compound could be a candidate for further study. This steroid in known to be released in the urine of reproductive male Mozambique tilapia (Oliveira *et al.*, 1996) a species that employs a similar male nest guarding reproductive strategy (Bruton & Boltt, 1975) to that used by topmouth gudgeon. Behavioural studies are required to provide evidence whether these compounds have a role in reproductive chemical communication in topmouth gudgeon.

Investigations testing EOG responses to specific High Performance Liquid Chromatography (HPLC) retention time fractions isolated from male conditioned water have been used as an aid to identify specific active compounds (Li *et al.*, 2002; Belanger *et al.*, 2004). Specifically, Li *et al.*, (2002) found that a time fraction resulted in high EOG magnitude responses in females and subsequent behavioural analysis enabled the sex attractant 7α-12α,24-trihydroxy-5α-cholan-3-one 24 sulphate to be identified. As this current investigation identified large magnitude EOG responses to reproductive male and reproductive female conditioned water in females, subsequent HPLC separation could be used

in conjunction with behavioural studies (chapter 5) with the aim of identifying specific active compounds.

The results of this current study are in agreement with other investigations using species that employ male nest guarding as a reproductive strategy. A strong EOG response to reproductive male conditioned water also occurs in the Mozambique tilapia (Frade *et al.*, 2002) and the round goby (Belanger *et al.*, 2004). Also, in agreement with this current investigation a lesser EOG response occurred to non reproductive males in the round goby (Belanger *et al.*, 2004) and in subordinate male tilapia (Frade *et al.*, 2002) suggesting that recognition of male reproductive status has evolved in freshwater teleosts that employ a common male nest guarding reproductive strategy.

CHAPTER 8. DISCUSSION

8.1. INTRODUCTION

The aim of this study was to investigate reproductive chemical communication in the cyprinids topmouth gudgeon and sunbleak. The use of reproductive chemical cues in these species had previously been unstudied. Knowledge gained from understanding pheromone systems in fish can be integrated into existing knowledge (the majority learnt from the study of insect pheromones) to formalise a more comprehensive understanding of chemical communication usage in animals. The shared use of a specific compound as a chemical cue is common in terrestrial organisms (see Wyatt, 2003) and studies concerning fish pheromones enable the independent evolution of these compounds in the animal kingdom to be further explored. In particular, compounds used as chemical cues could be different in fish than that of terrestrial organisms due to their aquatic environment, where solubility and not volatility is important for the transfer of information (Wyatt, 2003). Some fishes make good model species as they can be easier to obtain and maintain in the laboratory than other animals. As olfactory functions (mediation of similar key behaviours) (Sorensen *et al.*, 1998) and olfactory processing appears similar in all animals (Hildebrand & Shepherd, 1997), pheromone research using fish provide a good model for studying olfactory systems in vertebrates.

Topmouth gudgeon and sunbleak are unusual among fish, in employing nest-guarding reproductive strategies. Whilst much is known of the sex pheromone systems in species that employ a scramble spawning reproductive strategy (see chapter 1 and a review by Burnard *et al.*, 2008), research regarding fish species that use different reproductive strategies such as male nest guarding is currently limited. This study, which investigated the use of chemical cues in two species that employ two different forms of male nest guarding, provides a new perspective on reproductive chemical communication in teleosts. Reproductive

chemical communication in the two study species not only differs from species that employ scramble spawning but intriguingly, the two study species clearly have two distinct forms of reproductive chemical communication as a consequence of the two different versions of male nest guarding employed (see section 8.4).

An understanding of reproductive chemical communication in the two study species also has important implications for conservation. Sunbleak are an endangered species that is in rapid decline throughout Europe (Lelek, 1987; Gozlan *et al.*, 2005). Knowledge gained concerning reproduction in the species could have a role in preventing the extinction of the species. This is particularly true if captive breeding programmes are established. Primer pheromones could be used to induce sperm production for example, as suggested by Stacey *et al.*, (1994) for carp aquaculture. This could provide a less stressful and cost effective alternative to hormone injections (Wyatt, 2003).The first requirement for such study is the identification of a sex pheromone system.

In the UK, over 50% of introduced fish species employ parental care (Maitland, 2000). Furthermore, in England 44% of introduced fish species are nest guarders (DAFF, 2002). Therefore study concerning reproductive chemical communication in nest guarding species could realise pheromone control as an important tool in their management. Topmouth gudgeon are classed internationally as an invasive pest (see Gozlan *et al.*, 2002). Pheromones have already been used to control terrestrial insect and a number of studies have shown that the concept of controlling aquatic pest species using pheromones has potential (see Corkum, 2004 for a review). The first potentially viable application of sex pheromones appears to be in the sea lamprey (Li *et al.*, 2002) where initial projects have had great success in trapping sexually mature females using traps that contained sexually mature males (Johnson *et al.*, 2005. The first requirement for this potential to be realised in topmouth gudgeon is the identification of a sex pheromone system in the species. Subsequent study can then identify

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the precise functions behind specific chemical cues and the compounds that are used, to determine whether the application of pheromones as a control measure is viable in conservation management.

8.2. REPRODUCTIVE CHEMICAL CUES IN TOPMOUTH GUDGEON *PSEUDORASBORA PARVA*

This investigation determined for the first time responses to reproductive chemical cues in topmouth gudgeon. The results of this study strongly suggest that searching behaviour is induced in reproductive females during exposure to reproductive male released chemical cues. This is converse to the sex pheromone system in documented species that use scramble spawning and in particular that of the most well studied model, the goldfish. The similarities in chemical communication (evoked responses) between species that have a similar reproductive strategy raises the possibility that there are a number of 'blue prints' for which chemical communication in all teleosts could be applied, based on their reproductive mode (see a review by Burnard *et al.*, 2008). This hypothesis requires further testing which could only be achieved with characterising the sex pheromone systems of a greater number of species. In addition to induced searching behaviour, this study also suggests that courtship display behaviour is induced in reproductive females during exposure to reproductive male conditioned water. Further study is required to conclude that receptive females do display in the presence of reproductive male released cues. This could be achieved by comparing hormone levels and egg histology in females that headstand and females that do not headstand. In the goldfish for example, oocyte maturation is induced by a dramatic luteinising hormone surge (Sorensen *et al.*, 1998).

In other studies concerning the use of chemical communication in invasive species such as the round goby (Belanger *et al.*, 2004) and sea lamprey (Li *et al.*, 2002) induced

searching behaviour in reproductive females was coupled with attraction to the odour source. Therefore, chemical cues released by males could potentially be used in pheromone traps (Jonhson *et al.*, 2005; Corkum *et al.*, 2006). This current investigation did not determine an attraction to the odour source by reproductive females and therefore suggests that male released chemical cues in topmouth gudgeon would not be able to facilitate the capture of females in pheromone traps. However this hypothesis requires further testing in a bioassay specifically designed to investigate attraction to a pheromone source (see section 8.3).

This study was the first to demonstrate that reproductive cues released by male topmouth gudgeon can be successfully extracted and isolated using a combination of solid phase extraction (SPE) and high performance liquid chromatography (HPLC). This is essential for future study that aims to identify the compound(s) used as reproductive chemical cues. In addition, freezing the product (used if the compound is pooled) did not cause any detectable deterioration of the cue. This investigation has shown that the cue released by male topmouth gudgeon is not UV-active and can only be identified on HPLC peak by its retention time, which will require a strict reproduction of the protocols developed here in any further study aiming to identify the signal. Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy of an active isolate did show some weak signals but the intensity was not strong enough to be interpretable.

8.3. REPRODUCTIVE CHEMICAL CUES IN SUNBLEAK *LEUCASPIUS DELINEATUS*

This study is the first to document the occurrence of reproductive chemical cues in sunbleak. The findings demonstrate that reproductive females respond to a cue released by reproductive males. The existence of such a cue is expected when placed in the context of the reproductive strategy employed by sunbleak. In this species, females are enticed to lay

eggs in nests that males guard (Gozlan *et al.*, 2003a). The behaviour induced in reproductive females by reproductive male cues strongly suggests that the cue has a releaser function in the species, specifically to induce searching behaviour. The same type of response was also induced in reproductive male sunbleak during exposure to cues released by female sunbleak and reproductive males also respond to cues released by other reproductive males. Sunbleak employ allopaternal care (Gozlan *et al.*, 2003a), therefore it is expected that the cue released by reproductive males would be relevant to other reproductive males. This hypothesis could be further examined by testing the response of males to cues released by other males in other species that use allopaternal care (e.g. fathead minnow). The response of reproductive males to cues released by other reproductive males, strongly suggest that searching behaviour is evoked in male sunbleak during exposure to reproductive male cues.

Whilst the results of this investigation strongly imply that releaser type searching responses are induced by chemical cues in both test species, this hypothesis requires further study in a functional bioassay which would provide a greater understanding of animal's response to the cue in a natural system. Specifically, responses to odour derived from only one donor should be tested in a flow through system (see chapter 4, section 4.4). At present therefore, it can only be concluded with certainty that generic responses in swimming behaviour are evoked by reproductive chemical cues. Nevertheless that chemical cues have a role in the reproduction of both species can not be refuted.

8.4. COMPARISION OF THE INDUCED RESPONSES IN SUNBLEAK *LEUCASPIUS DELINEATUS* AND TOPMOUTH GUDGEON *PSEUDORASBORA PARVA* AND DISCUSSION OF INTER SPECIFIC RESPONSES

Induced behavioural responses in females during exposure to reproductive male conditioned water occurred in both species. Releaser type searching responses appear to be the same in both species. However there are clear differences in responsiveness between the species. In particular male sunbleak responded to water conditioned by other reproductive males, suggested to relate to the specific form of reproductive strategy (allopaternal) used by the species (chapter 4). Also, in sunbleak behavioural responses in reproductive males were evoked by reproductive female chemical cues. This is interesting, as both topmouth gudgeon and sunbleak males find reproductive females in the same manner (Maekawa *et al.*, 1996; Gozlan *et al.*, 2003a). Specifically, males of both species leave their nest to search for available females. Further study is required to determine why the responses to female cues differ in these species that appear to have a similar mode for finding mates. This phenomenon could also be researched in other species that use the same reproductive strategy as sunbleak (such as fathead minnow) to examine the possibility that responses to female cues in males is a trait of sex pheromone systems that use allopaternal care.

The induced responses in reproductive sunbleak to cues released by reproductive topmouth gudgeon are important in understanding sex pheromone systems. This study raises the possibility that cues released by topmouth gudgeon are confused with conspecific reproductive signals. The precise reason for this phenomenon (components of the signal are the same or the receiver makes mistakes) is a subject that warrants further investigation. The reason for this interspecific communication might allow an actual example to be applied to

the existing accepted hypothesis regarding the use of multi compound chemical cues in teleosts (Sorensen & Scott, 1994).

Due to the induced responses by heterospecific chemical cues shown in this study, the notion of 'pheromone pollution' requires investigation. Here, heterospecific chemical cues could disrupt reproduction between individuals in species that have had no previous exposure to them (i.e. have not co-evolved). The decline of sunbleak populations coincides with topmouth gudgeon introduction (see Gozlan *et al.*, 2005) and sunbleak spawning has been shown to be inhibited when exposed to reproductive topmouth gudgeon conditioned water (Gozlan *et al.*, 2005). The inhibition of spawning is thought to be caused by an intracellular parasite (Gozlan *et al.*, 2005). However this current investigation has shown that signals released by reproductive topmouth gudgeon induce responses in reproductive sunbleak only (not non reproductive). Further investigation is therefore warranted to determine if reproductive cues released by topmouth gudgeon impact on spawning in sunbleak. This subject has implications for native fauna due to the introduction of non native species.

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APPENDIX I. PUBLICATIONS

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REVIEW PAPER

The role of pheromones in freshwater fishes

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The study of fish pheromones is particularly relevant because of the conserved nature of chemoreception in vertebrates. However, most fish pheromone systems remain unstudied. All the major known pheromones of freshwater fish and their associated behaviours were reviewed. Importantly, those studies that have demonstrated the connection between behaviour and pheromones in freshwater fishes have resulted in a wide range of applications in management. For example, pheromones released by the sea lamprey *Petromyzon marinus* have a practical function in pheromone traps, showing how chemical communication can be used in the management of invasive species. Future research on fish pheromones should include olfactory systems in a wider range of species testing the possibility that a few distinct models could be applied to the all fishes. Progress in research on fish pheromones should include a closer collaboration with other research fields such as evolutionary biology to allow a better understanding of fish pheromones systems divergence and mate selection where correlation between phenotypic dominance and pheromone production is still largely ignored. Finally, the example of pheromone interaction between an invasive species topmouth gudgeon *Pseudorasbora parva* and a native endangered species sunbleak *Leucaspius delineatus* is provided to illustrate the concept of pheromone pollution that assists its establishment in a novel ecosystem. © 2008 The Authors
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Key words: chemoreception; communication; induced behaviour; invasive species.

INTRODUCTION

Smell plays an important role in communication for many animals. Surprisingly the first pheromone was only discovered in the past 50 years (Butenandt *et al.*, 1959). Only more recently have the chemicals responsible in aquatic systems been the subject of research (Colombo *et al.*, 1980). The latest definition of a pheromone accounts for new research (using teleost fish) on chemical communication between individuals. Notably, it is now accepted that pheromones can exist as a number of different compounds in vertebrates as well as in invertebrates

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(Poling *et al.*, 2001) and that specialization of the odour(s) to play a role in chemoreception is not required (Stacey & Sorensen, 2002). Pheromones are therefore defined as 'an odour or mixture of odours released by the sender that evokes in the receiver(s) adaptive, specific, and species-typical response(s), the expression of which need not require prior learning or previous experience' (Sorensen & Stacey, 2004).

The difficulty in accurately replicating concentrations of pheromones in laboratory and field experiments means that studying pheromone communication systems is challenging (Bentley & Watson, 2000). Nevertheless, chemical signals as an effective means of communication have been shown for a diverse range of taxa (Brönmark & Hansson, 2000; Martín & López, 2000; Kikuyama *et al.*, 2002; LeMaster & Mason, 2002; Brennan & Keverne, 2004). Not only is olfaction used by a wide range of organisms but a large number of different key behaviours are chemically mediated. This includes predator avoidance (Friesen & Chivers, 2006), migration (Sorensen *et al.*, 2005), shoaling (Mann *et al.*, 2003) and reproduction (Kobayashi *et al.*, 2002).

The active compounds and their metabolites used in olfaction are in some instances common for a number of taxa (Rasmussen *et al.*, 1996). This suggests that olfactory systems have evolved slowly and separately a limited number of times. Because of this, the study of chemical communication in early vertebrates, such as fish, is relevant to the entire animal kingdom. Knowledge of these pheromone systems could be applied to other animals that have more complex behaviour patterns, which would otherwise be difficult to interpret. This is particularly relevant in vertebrates where the components of the olfactory system (anatomical, cellular and biochemical) have remained highly conserved through evolution (Stacey *et al.*, 2003).

This review is focused on the pheromone systems of fish, and how these pheromone systems relate to a wide range of behaviours as well as providing some perspectives for aquatic conservation. Although research has advanced in the last couple of decades, identification of specific compounds used by a large number of species is required. In addition to being a blueprint for pheromone systems in vertebrates, there are wider applications of pheromone-related research in fish. Knowledge of the chemical signals associated with migration, predator avoidance and reproduction is now researched for its potential use in the control of invasive species. Here, attracting individuals into pheromone-laden traps is a viable option. Pheromones could also be a significant factor in determining mate selection in individuals, which provides opportunities for novel study in evolutionary ecology.

PHEROMONE-MEDIATED BEHAVIOUR

The nature of the aquatic environment lends itself to chemical communication. The high solubility of some pheromones in water can mediate the external transfer of information over large distances. While visual signals are very important in communication, poor visibility can render them obsolete. This has led to the evolution of chemical signals that act as cues for a range of behaviours in fish that can work in conjunction with other stimuli (*i.e.* visual and sound). Most pheromones that have been chemically identified to date are

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associated with reproduction (Table I), while little is known about the chemical structure of pheromones involved in other behaviours.

SPECIES SPECIFICITY

Generally it is accepted that closely related fish species have similar pheromone systems (compounds and effects) and distantly related species have dissimilar ones. This seems to be the case at least in hormonally derived sex pheromones (Irvine & Sorensen, 1993). For example, the common carp *Cyprinus carpio* L. and goldfish *Carassius auratus* (L.) appear to share common steroidal pheromone systems. Sensitivity to 17α,20β-dihydroxy-4-pregnen-3-one, 17α,20βP-sulphate and androstenedione occurs in both species (Irvine & Sorensen, 1993). Similar results are shown regarding sensitivity to 17α,20β-dihydroxy-4-pregnen-3-one and prostaglandin $F_{2\alpha}$ (PGF) in both goldfish and crucian carp *Carassius carassius* (L.) (Bjerselius & Olsen, 1993).

In mate choice, sex pheromones are used to attract or distinguish between potential conspecific mates. However, if two species have very similar sex pheromones, heterospecific mates also may be attracted and lead to hybridization if inbreeding occurs. In many freshwater fish species such interbreeding does occur (Verspoor & Hammart, 1991; Leary *et al.*, 1995; Hänfling *et al.*, 2005) and could be explained by both species having a mating response to the same pheromonal system (Bjerselius & Olsen, 1993; Irvine & Sorensen, 1993). This is well illustrated with hybridization between brook trout *Salvelinus fontinalis* (Mitchill) and brown trout *Salmo trutta* L. which both have equal sensitivity to PGF and its derivatives (Essington & Sorensen, 1996).

Given the limited number of compounds used as sex pheromones, hybridization between closely related species that share sex pheromones could be expected to be more common than observed. However, it is likely that there are a variety of precursors to reproduction and the event is not subject to one underlying factor. Other signals play a significant role in orchestrating spawning, including visual, auditory, tactile and electrical signals (Irvine & Sorensen, 1993; Olsén *et al.*, 2000). Differences in the timing of the mating season could also be a determinant to hybridization success.

According to evolutionary principles, it is expected that sympatric species would possess different sex pheromones. However, this is not always the case. Males of both Atlantic salmon *Salmo salar* L. and *S. trutta* show physiological responses to ovarian fluid and the urine of conspecific and heterospecific females (Olsén *et al.*, 2000). Despite showing a similar response to some chemicals, fishes can also discriminate between specific mixtures of compounds (Poling *et al.*, 2001) and a slight variation in the mixture could be enough to avoid hybridization between closely related species (Sorensen & Scott, 1994). This is particularly valid for the use of prostaglandins where its almost universal action would mean that an individual would not be able to discriminate between conspecifics and heterospecifics (Stacey & Cardwell, 1995).

While there is still some controversy as to whether alarm signals should be classed as pheromones or alarm substances (Magurran *et al.*, 1996; Smith, 1997; Hartman & Abrahams, 2000), they are important chemical signals used by fish (Wisenden *et al.*, 2004). This review conforms to the terminology used

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TABLE I. Known pheromone systems in freshwater fishes

Compound	Species	Effect	References
Prostaglandins			
Prostaglandin $F_{2\alpha}$	Atlantic salmon (*Salmo salar*)	Male priming	Moore & Waring (1996)
	Arctic charr (*Salvelinus alpinus*)	Female attractant and elicit spawning behaviour	Sveinsson & Hara (1995)
	Brown trout (*Salmo trutta*)	Female prespawning behaviour	Laberge & Hara (2003)
	Goldfish (*Carassius auratus*)	Elicit male sexual behaviour	Kobayashi *et al.* (2002)
	Lake whitefish (*Coregonus clupeaformis*)	Increased locomotor activity in males and females	Laberge & Hara (2003)
13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ ($F_{2\alpha}$ metabolite)	Cobitid loach (*Misgurnus anguillicaudatus*)	Elicits male sexual behaviour	Ogata *et al.* (1994)
	Brown trout (*S. trutta*)	Increased locomotor activity in males and females	Laberge & Hara (2003)
15-keto-prostaglandin $F_{2\alpha}$ ($F_{2\alpha}$ metabolite)	Goldfish (*C. auratus*)	Elicit male sexual behaviour	Kobayashi *et al.* (2002)
	Lake whitefish (*C. clupeaformis*)	Increased locomotor activity in males and females	Laberge & Hara (2003)
	Atlantic salmon (*S. salar*)	Male priming	Moore & Waring (1996)
Steroids un-conjugated			
Etiocholanolone	Round goby (*Neogobius melanostomus*)	Increased ventilation rate in males and females	Murphy *et al.* (2001)
		Female attractant (possible)	Arbuckle *et al.* (2005)
11-ketotestosterone	Round goby (*N. melanostomus*)	Female attractant (possible)	Arbuckle *et al.* (2005)
17,20β-P	Rainbow trout (*Oncorhynchus mykiss*)	Unknown effect in male and female	Vermeirssen & Scott (1996)
	Goldfish (*C. auratus*)	Elicit male sexual behaviour and physiological priming	Kobayashi *et al.* (2002)
	Roach (*Rutilus rutilus*)	Unknown effect in males and females	Lower *et al.* (2004)

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TABLE I. Continued

Compound	Species	Effect	References
Testosterone	Three spot gourami (*Trichogaster trichopterus*)	Unknown male effect	Becker *et al.* (1992)
	Yellowfin baikal sculpin (*Cottocomephorus grewingki*)	Elicit female spawning behaviour	Katsel *et al.* (1992)
Estrone	Round goby (*N. melanostomus*)	Increased ventilation rate in male	Murphy *et al.* (2001)
17β-estradiol	Round goby (*N. melanostomus*)	Increased ventilation rate in male	Murphy *et al.* (2001)
Steroids conjugated			
Etiocholanolone (sulphated)	Round goby (*N. melanostomus*)	Female attractant (possible)	Arbuckle *et al.* (2005)
Etiocholanolone (glucuronidated)	African catfish (*Clarias gariepinus*)	Female attractant (possible)	Resink *et al.* (1989)
17,20β-P (sulphated)	Goldfish (*C. auratus*)	Elicit male sexual behaviour	Kobayashi *et al.* (2002)
	Rainbow trout (*O. mykiss*)	Unknown effect in males and females	Vermeirssen & Scott (1996)
	Hill trout (*Barilius bendelisis*)	Male priming pheromone	Bhatt & Sajwan (2001)
17,20β-P (glucuronidated)	Rainbow trout (*O. mykiss*)	Unknown effect in males and females	Vermeirssen & Scott (1996)
	Roach (*R. rutilus*)	Unknown effect in males and females	Lower *et al.* (2004)
	Zebrafish (*Brachydanio rerio*)	Induces ovulation in females	Van Den Hurk & Resink (1992)
Dehydroepiandrosterone (glucuronidated)	Round goby (*N. melanostomus*)	Increased ventilation rate in males and females	Murphy *et al.* (2001)

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TABLE I. Continued

Compound	Species	Effect	References
Other			
7α-12α,24-trihydroxy-5α-cholan-3-one 24 sulphate (bile acid)	Sea lamprey (*Petromyzon marinus*)	Female sexual attractant	Li *et al.* (2002)
Petromyzonamine disulphate	Sea lamprey (*P. marinus*)	Component of migratory pheromone	Sorensen *et al.* (2005)
Petromyzosterol disulphate	Sea lamprey (*P. marinus*)	Component of migratory pheromone	Sorensen *et al.* (2005)
Petromyzonal sulphate	Sea lamprey (*P. marinus*)	Component of migratory pheromone	Sorensen *et al.* (2005)
Nitrogen oxide functional group	Ostariophysan fishes	Alarm cue	Brown *et al.* (2000)

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in the latest research (Barreto & Hoffmann, 2007; Chivers *et al.*, 2007) and classes them as alarm substances. The species specificity of alarm substance has been the subject of much study (Mirza & Chivers, 2001; Leduc *et al.*, 2003; Kelly *et al.*, 2006). Many fishes use the damage-released alarm substance (called Schreckstoff) of heterospecifics to cue the implication of a predator avoidance mechanism (Wisenden *et al.*, 2004). Chivers *et al.* (2002) suggest that many sympatric prey species share alarm substance or have ones that are nearly identical. In addition, there is evidence to indicate that phylogenetically related invertebrate species are more likely to respond to alarm substances from closely related species, *cf.* more distantly related species (Fässler & Kaiser, in press).

Because of the survival benefits conferred to individuals that recognize alarm substance, some heterospecifics that do not possess a common cue learn the alarm signals of sympatric species (Mirza & Chivers, 2001). This occurs when sympatric alarm cues are detected in combination with the conspecific alarm cues. In addition, predators use these signals as foraging cues (Brown *et al.*, 2001). Ostariophysan fishes, for example, release a common alarm chemical contained in specialized club skin cells (Chivers & Smith, 1998).

PHEROMONE-MEDIATED BEHAVIOUR AND PHYSIOLOGICAL RESPONSES

Pheromone-mediated behaviour and physiological responses are diverse and occur in a variety of freshwater fishes. The responses induced by pheromones can be divided into releaser and primer types (Wilson & Bossert, 1963; Stacey & Sorensen, 2006). Releaser responses are rapid behavioural changes and primer responses are slower physiological effects. Even though different species can produce and release the same pheromone, the responses they induce may vary. For example, conjugated (sulphated) 17α,20β-dihydroxy-4-pregnen-3-one induces releaser responses in male *C. auratus* (Kobayashi *et al.*, 2002) and primer effects in hill trout *Barilius bendelisis* (Hamilton) (Bhatt & Sajwan, 2001). Also, a pheromone is not restricted to a specific sex. In the Arctic charr *Salvelinus alpinus* (L.), it is the males that release PGF to elicit a behavioural response in females (Sveinsson & Hara, 1995), while in the *S. salar* females release PGF to prime males (Moore & Waring, 1996).

There are numerous examples of induced behavioural responses to reproductive pheromones in freshwater fishes (Cole & Smith, 1992; Zheng *et al.*, 1997; Yambe & Yamazaki, 2001; Sorensen *et al.*, 2004). In the round goby *Neogobius melanostomus* (Pallas), for example, attraction of females to water conditioned by reproductive males has been illustrated with increased swimming velocity, increased time spent near the odour source and a change in swimming pattern (Gammon *et al.*, 2005). Induced physiological responses to reproductive pheromones in freshwater fishes are also well known (Zielinski *et al.*, 2003; Olsén *et al.*, 2006). Such physiological effects include increased milt production (Stacey & Sorensen, 1986), increased gill ventilation (Belanger *et al.*, 2006) and olfactory response through electro-olfactogram (EOG) (Murphy *et al.*, 2001).

Behaviour associated with alarm and antipredator cues in freshwater fishes include increased shoaling, dashing and freezing (Brown & Dreier, 2002), fin flicking (Brown *et al.*, 1999), predator avoidance (Friesen & Chivers, 2006) and altered

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inspection behaviour (Brown & Dreier, 2002). Glowlight tetras *Hemigrammus erythrozonus* (Durbin) have been shown to alter their inspection behaviour when exposed to predators fed with tetras compared with their response to predators fed with swordtails *Xiphophorus birchmanni* Lechner & Radda (an unrecognizable heterospecific alarm substance). Inspection behaviour directed at the head of the predator was reduced in favour of tail-end inspections (Brown & Dreier, 2002).

Behavioural responses induced by alarm substances are not restricted to prey. Fathead minnows *Pimephales promelas* Rafinesque exhibit a fright response to, and actively avoid areas that contain the faeces of pike *Esox lucius* L. that have recently been fed fathead minnows (Brown *et al.*, 1995*a*). It is known that *E. lucius* spend a greater proportion of time in the area where they were fed but the majority of their faeces are deposited away from the foraging area. *Esox lucius* therefore is able to counter the effects of being labelled as a predator by the alarm substance of the prey species using this behaviour (Brown *et al.*, 1995*b*).

Freshwater fishes are able to recognize kin members through waterborne odours (Stabell, 1984; Hiscock & Brown, 2000; Courtenay *et al.*, 2001). It is believed that close relatives will stay together so that they can benefit from nepotistic behaviour. *Salmo salar* and rainbow trout *Oncorhynchus mykiss* (Walbaum) juveniles are less aggressive to siblings than non-kin members and also have smaller territories when located next to related siblings (Brown & Brown, 1993; Brown *et al.*, 1996). The ability to discriminate between conspecifics by odour has other implications in behaviour. During the winter, *S. salar* preferentially associate with non-kin possibly because this reduces competition among siblings (Griffiths *et al.*, 2003). The histocompatibility complex is used by fish in mate choice decisions (Milinski, 2003). It is possible that females avoid close relatives, choosing instead males with non-matching, complementary immune genes that impart strong defence against parasites and disease.

The evolution of alarm substance has been a subject of much debate (Chivers *et al.*, 1996; Wisenden & Smith, 1998; Wisenden, 2000). The benefit to the donor in releasing alarm cues is the attraction of a second predator increasing the handling time of the first predator and the probability that the prey species will escape (Chivers *et al.*, 1996). However, other authors (Smith, 1997) have argued that alarm signals are released to warn other shoal members of predator threat. This would only be applicable if shoal members were composed of kin members, of which there is presently scant evidence. The nitrogen oxide functional group responsible for alarm substance (Brown *et al.*, 2000, 2003; Kelly *et al.*, 2006) is released as a by-product of damaged skin cells and has an immune function (Chivers *et al.*, 2007). It is now proposed that fishes have evolved an antipredator mechanism initiated by this cue because of the benefits of recognizing an odour associated with a nearby predation risk (Chivers *et al.*, 2007). The evolution of Schreckstoff substance as an alarm cue therefore mirrors the accepted evolution of hormonal reproductive pheromones proposed by Stacey & Sorensen (1991).

THE USE OF PHEROMONES IN CONSERVATION

The biodiversity of aquatic ecosystems is vulnerable to the introduction of non-native species (Sala *et al.*, 2000), posing a threat through predation of native species, resource competition, introduction of new diseases (Gozlan *et al.*, 2005)

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or alteration of the environment (Manchester & Bullock, 2000). Any technique that could help mitigate this problem is a potentially important tool in conservation. Pheromones play a vital role in the reproduction of some invasive species, such as the *N. melanostomus* and sea lampreys *Petromyzon marinus* L.

Originating from the Black and Caspian Seas, the *N. melanostomus* has already been reported in the Mississippi River basin (Jude *et al.*, 1992) and has spread to the Great Lakes. This could lead to a loss of biodiversity because of competition for resources in species such as mottled sculpin *Cottus bairdi* Girard (Dubs & Corkum, 1996) and egg predation in lake trout *Salvelinus namaycush* (Walbaum) (Chotkowski & Marsden, 1999) and lake sturgeon *Acipenser fulvescens* Rafinesque (Nichols *et al.*, 2003).

Research on the reproductive pheromones used by the *N. melanostomus* is currently advancing (Corkum & Belanger, 2007). In the laboratory, mature female *N. melanostomus* increase ventilation rate when exposed to odours released by mature males (Murphy *et al.*, 2001). EOG analysis on >100 synthetic steroids and prostaglandins found that 19 steroids elicited a response. Attraction of females to water conditioned by males (shown as increased time spent near the source) is also documented (Gammon *et al.*, 2005). The potential for pheromone use in the control of this species therefore appears to be a possibility.

The North American Great Lakes *P. marinus* control programme has enjoyed success by the application of an integrated pest management (IPM) approach. Here, different life stages of the non-native pest species are targeted simultaneously by a variety of methods including the use of toxins and the introduction of sterile males.

The high fecundity of *P. marinus* and the damaging effect on native fishes has led to the search for more methods of control. Application of the recently discovered 7α,12α,24-trihydroxy-5α-cholan-3-one 24-sulphate (3-keto-petromyzonal sulphate) (Li *et al.*, 2002) may prove successful. This is an active component of a potent pheromone released by spermiating males that induces searching behaviour in ovulated females. As it is a bile acid and more water-soluble than steroids, it can be detected from a greater range (up to 65 m; Li *et al.*, 2002). This makes it an ideal candidate for use in IPM and could be used to trap mature females. Application could result in a shift in the sex ratio of the species causing severe competition for mates (Corkum, 2004).

Field trials using baited traps containing either spermiating male *P. marinus* or washings from spermiating males have enjoyed considerable success in trapping females (Johnson *et al.*, 2005, 2006). The capture of female *P. marinus* using chemical cues alone is therefore viable. The untested application of a synthetic sex pheromone to trap this species is a new prospect for research. If successful, high concentrations of the active components would be available thus potentially providing a more ethical and effective trap than that which contains live specimens. The success of a synthetic pheromone would of course be dependent on the cost of manufacture and the stability of the pheromone.

However, although *P. marinus* is invasive in the Great Lakes, its status is considered to be vulnerable in Europe (Renaud, 1997). The mix of three steroidal compounds released by larvae that constitutes a *P. marinus* migratory pheromone [and potentially used to control this species in the Great Lakes (Sorensen *et al.*, 2005)] could also be used to restore European population by guiding

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adults to suitable spawning streams. Low concentrations (final concentration of 5×10^{-13} mol l^{-1}) of this migratory pheromone have been found to be sufficient to attract 90% of actively migrating *P. marinus* to a branch of a river where it was released (Wagner *et al.*, 2006).

A component of this migratory pheromone, petromyzonal sulphate, has been identified in the gallbladders of different species of European and North American lamprey (Fine *et al.*, 2004). This indicates that larval petromyzontid lampreys respond to an evolutionary conserved pheromone. The potential use of this pheromone could therefore be extended to include not only *P. marinus* control but also lamprey conservation worldwide. As the poisoning of threatened lamprey species occurs as a by-product of non-native lamprey control (Renaud, 1997), the potential use of pheromones in IPM as an alternative to lampricides would also directly benefit lamprey conservation.

DISCUSSION

Chemical signalling occurs in a wide and diverse number of fish species. Although research in this field has progressed, there is a lot of scope for further study. The vast majority of fish species have yet to be investigated. It is unknown whether the best understood reproductive signalling mechanism, that of the *C. auratus*, is a common mechanism for reproductive pheromone signalling in fish. Consequently, it is not known whether the knowledge of this basic model species could be applied in a management–conservation setting to the many species of fish in decline or under threat.

Greater understanding of pheromone activity can only be achieved through study of other fish species. A focus for future study could be to extensively detail the reproductive olfactory systems used by other orders of fish (as opposed to Cypriniformes) such as Perciformes or Salmoniformes. This would highlight how pheromones systems have diverged and give an indication of the extent to which olfactory systems are generic in freshwater fishes.

Another understudied aspect of pheromone research in fish is the importance of olfaction in determining mate selection. The knowledge of sexual selection is currently biased towards variables that can be defined visually, such as colour or display behaviour. While compatibility between individual three spined sticklebacks *Gasterosteus aculeatus* L. is now considered to be defined by odour (Milinski, 2003), the link between preference and olfaction is presently understudied. In *X. birchmanni*, it is suggested that a sex-specific chemical cue conveys information about male nutritional state and that females attend to this cue during mate choice (Fisher & Rosenthal, 2006). It is likely that potential mates can discriminate different aspects of quality that influence selection through chemical cues.

In mate selection, correlation between phenotypic dominance and pheromone production is still ignored, despite the potential to be an important parameter. Chemical signals may be a factor determining dominance between individuals, in addition to known factors such as size and aggression. Male Mozambique tilapias *Oreochromis mossambicus* (Peters) increase their urination rate in the presence of rival males, perhaps advertising their dominant status (Almeida *et al.*, 2005). It is suggested that as intruders may flee a resident's territory before fighting commences, chemical signals act to modify the aggressiveness

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of the intruder (Almeida *et al.*, 2005). Future research should consider chemical signals as a component influencing hierarchy and selection within populations in addition to known variables or parameters.

While a large number of fish species use pheromone signalling in reproduction, olfactory response in other events, such as mediating predator avoidance, is not always fully understood. The degree of olfactory specialization used to transfer key information undoubtedly varies in different species depending on their life history. Investigation into the relationship between the importance of the activity and degree of chemoreception specialization is required. This can also be applied to reproduction. It is possible that the different nature of courtship between species means that reliance on chemical communication varies.

Pheromone interaction between species, in particular in the context of non-native species introduction, is important as pheromone pollution could facilitate establishment by invasive species in a new environment. As common or similar pheromones are employed by different species (Irvine & Sorensen, 1993; Essington & Sorensen, 1996), disruption of courtship may result from non-native pheromones. Species-specific modification to separate similar pheromone systems and to prevent a generic response has not necessarily evolved because of geographical isolation. If a dominant member of a species uses chemical signals to modify behaviour among subordinates as suspected in topmouth gudgeon *Pseudorasbora parva* (Temminck & Schlegel), this could also affect the behaviour of individuals of other species. The case of breeding suppression of sunbleak *Leucaspius delineatus* (Heckel) by the introduction of *P. parva* is one example under investigation by the authors.

More and more pheromone research incorporates the use of the EOG technique. This allows the individual compounds that elucidate a response to be identified and isolated from mixtures of compounds. It also enables testing a wide range of chemicals for an active response. Compounds isolated from one species can be tested for a response on another. The effects of pollution on hormonal and pheromone activity can also be studied using this approach. EOG is therefore a necessary tool and provides greater insight and validation than that provided by behavioural experiments alone. Further study should now commonly incorporate EOG in combination with other techniques such as high performance liquid chromatography and behavioural assays to accurately map olfactory systems.

Pheromone signalling occurs throughout the animal kingdom. As pheromones have been identified in primitive fishes such as Elopiformes, it is expected that they are common among all species of fish (Stacey *et al.*, 2003). So the scope for further study in this field is vast. There is great potential to apply pheromonal research to aid in conservation management of freshwater fishes worldwide. The success of the few projects aiming to employ pheromones in this capacity is encouraging. It is clear that new advances in the field of fish behaviour and fish ecology will be heavily influenced by the knowledge gained from fish pheromones.

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