Anti-predator behaviour in the freshwater gastropod *Lymnaea stagnalis*

By

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Abstract

The freshwater gastropod *Lymnaea stagnalis* was used as a model organism to investigate the mechanisms employed by prey species to fine-tune anti-predator behaviour to match their environment. *Lymnaea stagnalis* was found to exhibit both genetic adaptation of innate responses and also induced responses to predator cues. Snails were also capable of responding to predation cues via associative learning dependent on recent experience. Constitutive responses were found to differ between populations depending on the predator regime that the population experienced in the wild. Artificial selection produced in only two generations a difference in the magnitude of response between high and low response selected lines equal to those seen between field populations in two generations. At the same time these selected lines maintained phenotypic plasticity and responded to exposure to predator cues during development. This developmental plasticity led to an increased response to predation cues in the low selected line equivilent to that in the high response selection line; a lack of induced change in behaviour in the high response selection line suggested a physiological limitation on the maximum anti-predator response. The response in the low selection lines indicates that plasticity in anti-predator behaviour could allow individuals with low innate responses to compensate with high levels of induced response. Finally, *L. stagnalis* was able to utilise alarm cues from prey guild members (i.e. other freshwater gastropods) to assess predation risk, a response that was dependent on the phylogenetic relationship between *L. stagnalis* and the species producing the alarm cue. However, this response was dependent on whether the species was found sympatrically (co-habiting the same water body) with *L. stagnalis*. Together, the rapid microevolution of constitutive responses in *L. stagnalis*, its ability to show induced responses and associative learning indicates that this species may be able to respond rapidly to a novel predation environment, and therefore allow colonistion of new habitats or identification of novel predators.

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Authors Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate committee.

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Chapter 1

Introduction

Predation has severe consequences for fitness, both in terms of the direct costs of being predated, but also indirect costs associated with induced responses to predator presence reducing potential for growth and reproduction (Lima and Dill 1990). As such, a prey organism will be under strong selection to respond appropriately to predation threats. The way in which an organism responds may include morphological, physiological and behavioural defences or changes in life history to avoid encountering predators. In this thesis I am interested in exploring the variety of different mechanisms an organism uses to fine-tune anti-predator behaviour.

Evolution of predator defence

Predator defence traits have evolved through strong selection pressure on prey organisms to respond to potential predators. Prey are considered to be ahead in the arms race between themselves and their predators due to a disparity in the fitness consequences of this interaction; for prey a predation event results in losing their lives, whereas for predators failing to consume a prey item is the loss of a meal (Dawkins and Krebs 1979). This disparity in evolutionary adaptation can be illustrated by the reciprocal plasticity occurring in one aquatic predator-prey system. The prey, a hypotrichous ciliate *Euplotes octocarinatus*, grows 'wings' to increase body size and reduce predation by a predatory ciliate *Lembadion bullinum*; in response to this induced defence *L. bullinum* increased cell and gape size to enable it to engulf the enlarged prey (Kopp and Tollrian 2003). This counter response is not fully adaptive as the defence is still effective in reducing predation rates, nor does it increase net energy intake for the predator due to the increase in energy required for the extra growth. This suggests that currently at least, *E. octocarinatus* is winning the evolutionary arms race (Kopp and Tollrian 2003). Morphological adaptation in prey to counter predation risk is thought to have shaped the evolution of several taxa, one example being the decrease in the proportion of gastropod taxa with weak shell designs co-occurring with an increase in shell crushing predators (Vermeij 1987).

Interactions between predators and prey species will be controlled by a number of traits of both the predators and prey, including life history, morphology, physiology and behaviour. Phenotypic integration between traits may occur under selection, where the values of different traits have been altered to provide the best selective response within given environmental conditions (Pigliucci 2003). However, this linkage of traits may reduce the adaptive potential of an organism under novel environmental conditions. There are frequently tradeoffs between defence traits and other aspects of organismal biology, for example *Lymnaea stagnalis* increases vulnerability to pathogens by exhibiting avoidance behaviour (Rigby and Jokela 2000), and predator induced traits in other taxa have been found to result in reductions in growth and reproductive potential (Crowl and Covich 1990, Van Buskirk 2000, McPeek 2004). Where tradeoffs exist the degree to which prey can adapt to predation risk may be limited.

The effect of predator species on prey evolution is currently not clear, however there is evidence that predator presence is driving trait divergence between populations (Laurila et al. 2006, Fisk et al. 2007), and also between allopatric ecotypes (Vamosi and Schluter 2004). There are also patterns of divergence within phylogenies which appear to suggest that adaptation to predator regime may drive divergence between closely related species

(Schluter 2001). The maintenance of this divergence may occur if species subsequently overlap in distribution and if the hybrids then demonstrate reduced fitness.

In aquatic systems chemicals released by predators appear to give the earliest warning system to prey species about their presence and identity. As such it seems surprising that predators have not evolved mechanisms to mask odour, in a similar manner to the way crypsis masks visual cues. Indeed, the only example of an apparent lack of chemical recognition of a predator in the aquatic environment is that of tailed frog tadpoles, *Ascaphus truei*, which respond to chemical cues from other fish predators, but fail to respond to cues from shorthead sculpin, *Cottus confusus*, possibly because the sculpin is masking its scent (Feminella and Hawkins 1994). Chemicals tend to be predator specific, so perhaps carry out other vital roles in allowing predator species to identify one another to allow mate recognition or establishment of territory. The fitness benefits associated with these other roles may outweigh the costs of letting prey species know they are present.

Ecology of predator defence

In predator-prey systems it is generally assumed that there is a link between predator and prey abundance, such that prey abundance limits predator abundance by limiting their food intake, and predators limit prey due to a positive relationship between prey abundance and the number of prey eaten (Morin 1999). In reality this link is more complex as predators are likely to take a number of different prey taxa in a system, such that both the predator identity and density and relative abundance and quality of other prey available will also affect this relationship. Other environmental variables including food availability to prey, substrate type and numerous other abiotic factors will also influence prey vulnerability (e.g. Ramcharan et al. 1992, Dodson et al. 1994).

Predators can significantly influence the community structure in which they live, and such predators have been termed 'key-stone predators' (Paine 1969). These are generally predators that target competitively dominant prey species, preventing them from out-competing the competitively inferior species which tend to be better at surviving predation threat due to evolutionary trade-offs (Paine 1969, Navarrete and Menge 1996, Morin 1999). This targeting of competitively dominant species may occur because these species are likely to be abundant in the habitat, and by targeting dominant species predators may lead to an increase in the diversity of prey taxa (Morin 1999). Predators that don't feed selectively may also alter community structure, but they tend to reduce the diversity of taxa rather than increase it. For example mosquito larvae, *Wyeomia smithii* feed on protists in pitcher plants, and tend to reduce protist diversity in plants where they are present (Addicott 1974). In this example there is little competition amongst protist and a generalist predator decreases the number of species by chance by reducing overall abundance.

Predators may also alter community structure by causing a switch in the type of prey taxa present. For example in freshwater aquatic systems large zooplankton tend to dominate in the absence of predatory fish, whereas small zooplankton dominate in the presence of predatory fish (Brooks and Dodson 1965). This is possibly due to competitive exclusion by large zooplankton, but evidence points towards selective feeding on large bodied plankton by predators and possibly also feeding of large zooplankton on smaller individuals (Dodson 1974). Trophic cascades may also affect species on other trophic levels than the predator and prey (Carpenter and Kitchell 1993).

For example predator consumption may result in fewer herbivores and consequently an increase in plant biomass (e.g. Brönmark 1994, Beklioglu and Moss 1998, Peacor and Werner 2001). Meta-analysis of freshwater trophic cascades suggests the presence of freshwater fish decreases the biomass of zooplankton, leading to an increase in phytoplankton biomass, though the response of phytoplankton was only very strong in a third of the studies reviewed (Brett and Goldman 1996).

The examples so far have outlined a few of the direct effects of predators through reductions in prey density within an ecosystem (density-mediated interactions), however indirect effects (trait-mediated interactions) may also occur via predator induced changes in prey traits. Changes in habitat use and feeding behaviour result in reduced impact of herbivores (e.g. Turner 1997, Werner and Peacor 2003), indeed the effects of indirect interactions may be greater than those of direct consumption on community dynamics (Preisser et al. 2005). Trophic cascades due to trait-mediated interactions may have both positive and negative effects on plant biomass by shifting the habitat use of herbivores, favoured plants may suffer reduced herbivory, whereas ones used as refuges from predators may experience increases in herbivory (Schmitz et al. 2004).

Chemical cues and predator-prey interactions

Chemical cues used by prey species may include predator cues, disturbance cues, alarm cues and dietary cues (Wisenden 2000). The response to alarm cues (particularly in combination with predator cues) is found in numerous taxa, suggesting that the ability to use such cues has adaptive benefits in many species. Chemical cues are likely to be available to potential prey species before contact with the predator occurs (Kats and Dill

1998), and hence may provide an early warning system of potential threat before visual or tactile cues are available.

Chemical cues may be used to identify a predator, for example the herbivorous gastropod, *Tegula funebralis*, crawls out of the water in response to predatory starfish, but not in response to non-predatory starfish (Yarnall 1964). When a prey species gains information about a predator they do not necessarily have any information about whether a predator has detected them (Kats and Dill 1998), or if the predator is currently foraging. Hence the use of alarm cues in combination with predator cues may provide information on the current foraging activity of that predator, and can be a reliable indicator of risk (Chivers and Smith 1998).

Frequently there is little, if any, response to alarm cues alone, potentially due to the lack of information contained about the nature of the threat, which may mean there is potential for the prey species to show inappropriate responses. Induced morphological responses to alarm cues alone are not found frequently, a review by Chivers and Smith (1998) could only find one example of an induced morphological response, in the crucian carp, *Carassius carassius* (Stabell and Lewin 1997). Life history characteristics appear more sensitive to alarm cues presented in isolation. Responses have been found in gastropods (Crowl and Covich 1990) and amphibians (Chivers and Smith 1998). However, what is clear from numerous studies is that alarm cues interact with cues from predators to produce the strongest responses in behaviour, morphology and life history in the majority of taxa studied (reviewed in: Dodson et al. 1994, Chivers and Smith 1998), and that in several cases this combination of responses is necessary to elicit a response.

In the aquatic environment the ability to utilise vision may be hindered by turbidity and habitat complexity, hence many aquatic species rely on chemical information to assess potential predator presence (Dodson et al. 1994, Kats and Dill 1998). The use of chemical cues has been found both in the identification of a potential predator (Chivers et al. 1996, Bernot and Turner 2001, Brown 2003), but can also be utilised to assess the threat posed by that predator (Kats and Dill 1998, Van Buskirk and Arioli 2002, Ferrari et al. 2006). Predator released kairomones (Dicke and Sabelis 1988) or disturbance and alarm cues from other aquatic organisms potentially give the earliest warning systems for prey species, and may be of particular importance to species with limited mobility compared to their predators (Wisenden 2000).

The term kairomone used in this thesis refers to chemicals released by one animal, received by another, that provides benefit to the receiver, but may be of no benefit to the sender (Dodson et al. 1994). The more general term 'cue' (when referring to chemicals released by tench or other predators) is also used interchangingly with kairomone. Alarm cue in this thesis always refers to chemicals released by damaged conspecifics; 'disturbance cue' is used as the terminology for signals given off by an animal in the presence of a predator without any damage having occurred.

Basis for behavioural responses

Behavioural modification in response to predator cues may occur at an innate level either through genetic adaptation to behave in a particular way on first encountering predator cues (Lively et al. 2000) or through transgenerational modification where parents induce developmental responses that alter the way an animal responds to predation threat (Agrawal et al. 1999). Alternatively, an organism may change behaviour via experience, exhibiting phenotypic plasticity during its development in response to environmental cues, either by increasing the level of response seen or altering type of response seen (Tollrian and Harvell 1999).

Diet information (i.e. cues released from a predator following consumption of the prey) or pairing of predator cues with damaged conspecifics can allow an organism to learn to recognise a potential predator through cue association or give an indication of current predator foraging activity. Prey can then adjust their responses appropriately. Increased responses to predator cues following experience of predation events has been noted in several aquatic invertebrates including: damselflies, *Enallagma* spp. (Chivers et al. 1996), crayfish (Hazlett et al. 2002), whelks, *Buccinum undatum* (Rochette et al. 1998) and a flatworm, *Dugesia dorotocephala* (Wisenden and Millard 2001). In these cases it was unclear as to whether the prey species were learning to identify a potential predator *per se*, or whether the association between cues was providing information about the perceived risk from the predator.

In freshwater systems a number of studies have demonstrated strong responses to predation cues (Reviewed in: Dodson et al. 1994, Chivers and Smith 1998, Lass and Spaak 2003, Werner and Peacor 2003), however many aquatic prey species occupy a wide variety of habitats and experience varying levels of predation threat, such that a single mode of behavioural response is unlikely to be an effective method of avoiding all potential predators. As such, it might be predicted that anti-predator behaviour would vary between populations with different predator regimes to closely match the type and level of predation threat present (McPeek 1990). Local adaptation to predation threat is likely to occur in relatively sedentary species with low dispersal abilities, whereas induced responses are more likely in species which have potential for high dispersal between generations, as parents and offspring are more likely to encounter alternate predator regimes (Kawecki and Ebert 2004). Adaptation may also be favoured where: i) plastic responses are costly if the lag between expressing one phenotype or its alternative is too far behind the changes in the environment (Padilla and Adolph 1996); ii) information gathering to produce the correct phenotype is costly (DeWitt 1998); or iii) costly responses to non-threatening species are likely (Langerhans and DeWitt 2002). Adaptation through natural selection has been shown to account for divergence between aquatic populations experiencing differing predation pressure (Cousyn et al. 2001, O'Steen et al. 2002, Meyer et al. 2006).

Alternatively prey may exhibit induced defences (Tollrian and Harvell 1999), a form of phenotypic plasticity, where the expression of the genotype varies depending on the environmental conditions (Bradshaw 1965). A meta-analysis of data from studies into the impact of predators on prey populations demonstrated that more than 50% of the impact on prey demographics from predators is due to induced defences, such as reduction of activity and feeding time, rather than direct effects from consumption (Preisser et al. 2005). Genetic variation for plasticity exists (Stinchcombe et al. 2004), and plastic responses can be selected for both for the mean response within a particular environment and also the degree of plasticity in response to alternate environments (Scheiner 1993, 2002, Garland and Kelly 2006). Behavioural responses tend to have a very short lag time between the expression of one phenotype and the next, and could therefore be very flexible in how they are expressed (Gabriel et al. 2005). Plasticity may occur during development, with the phenotype expressed in adulthood resulting from environmental cues earlier in life (Relyea 2001, Alvarez and Nicieza 2002) or environmental cues may effect the developing organism at any stage of development (Laurila and Kujasalo 1999, Laforsch and Tollrian 2004, Griffith and Sultan 2006).

Plasticity may confer advantage to organisms that have to deal with changes in their environment, or potentially enable them to survive in a novel environment (Parsons and Robinson 2006, Nussey et al. 2007). The ability to respond plastically to predatory threats may reduce the negative impact on fitness on encountering a novel predator, and therefore allow the animal to adapt further to increase fitness (Latta et al. 2007). However, there is also an increasing body of evidence that plasticity in a trait can also incur fitness costs (DeWitt 1998, Agrawal et al. 2002, Merilä et al. 2004, Stinchcombe et al. 2004, Teuschl et al. 2007), and may also lead to incorrect assessment of predation risk from organisms not previously encountered (Langerhans and DeWitt 2002, Schlaepfer et al. 2005).

As well as using conspecific alarm cue to recognise predation threat organisms can also respond to heterospecific cues through 'eavesdropping' (Stowe et al. 1995, Bradbury and Vehrencamp 1998). Eavesdropping on alarm cues from heterospecific intraguild members has been found in several species and may be used to provide additional information about potential predation risk (Stenzler and Atema 1977, Mirza and Chivers 2001a, Hazlett and McLay 2005, Schoeppner and Relyea 2005). In predator-prey systems, recognition of alarm cues from closely related species is not considered communication as there is no benefit to the signaller (Bradbury and Vehrencamp 1998), however it may be of benefit to the receiver to assess predation risk accurately and, hence, may reduce the high fitness costs associated with predation (Lima and Dill 1990). There is considerable evidence that alarm cues are conserved within phylogenetic groups (Pfeiffer 1977), with responses to alarm cues from closely related species generally being stronger than from those more distantly related (Snyder 1967, Stenzler and Atema 1977, Brown et al. 2003, Vilhunen and Hirvonen 2003), though the nature of the relationship between phylogenetic distance and response has only been investigated for the tadpole *Hyla versicolor* (Schoeppner and Relyea 2005). Snyder (1967) proposed that responses to alarm cues from heterospecific species sharing a common predator would be advantageous to a prey species regardless of taxonomic relationships. This theory would suggest that the effect of phylogenetic relatedness might be modified via the effects of sympatry or allopatry. There have been several studies which suggest that sympatry may modify responses to alarm cues from heterospecific species, however, these studies either used wild caught individuals and so could not rule out the effects of experience (Stenzler and Atema 1977, Chivers et al. 1997, Rochette and Dill 2000, Sullivan et al. 2003, Hazlett and McLay 2005) or used too few species to investigate any interaction with the effects of phylogenetic relatedness (Laforsch et al. 2006).

Aquatic Gastropods as prey

Aquatic gastropods have limited mobility relative to many of the predatory species they encounter; as such it might be predicted that they rely primarily on chemical cues to give early warnings in their assessment of predation risk. Aquatic gastropods have good chemosensory ability (Croll 1983), and have been shown to respond to a variety of predator-related chemosensory cues including conspecific and heterospecific alarm cues (Snyder 1967, Stenzler and Atema 1977) as well as showing differential responses to alternate predation threats both related to the type (Snyder 1967, Turner et al. 1999, Turner et al. 2000, Hoverman et al. 2005) and proximity (Turner and Montgomery 2003) of predators. Learning through cue association has also been shown to influence the degree of response to predator cues shown by aquatic gastropods (Rochette et al. 1998, Dalesman et al. 2006, Turner et al. 2006), and morphological responses have been demonstrated to be plastic (Trussell 2000a, Rundle and Brönmark 2001, Turner and Montgomery 2003, Cotton et al. 2004); the degree of such responses may also vary with resource availability such as food (Wojdak and Luttbeg 2005) or calcium concentration (Rundle et al. 2004).

The freshwater gastropod, the great pond snail, *Lymnaea stagnalis* (Gastropoda: Basommatophora) was used as a model prey organism in this study to investigate the mechanisms controlling anti-predator behaviour. This species was chosen as a model as it is known to respond both behaviourally and morphologically to predator cues (Snyder 1967, Rigby and Jokela 2000, Rundle and Brönmark 2001, Rundle et al. 2004), and although morphological responses are known to be induced by exposure to predator cues there is very little known about the way in which behavioural responses are controlled, other than they may be reduced when morphological defences increase (Rundle and Brönmark 2001, Rundle et al. 2004). It is relatively easy to obtain adult *L. stagnalis* from a variety of locations which differ in the predator regime they experience, and they may be reared through several generations in controlled laboratory conditions, producing a large number of offspring (see below). This ability to laboratory rear experimental animals meant that it was possible to explore the mechanisms controlling the behavioural responses seen, both in terms of the innate responses, and also how cue association learning or induced behaviours can alter these innate responses. In the following chapters I use *L. stagnalis* to address the various control mechanisms for anti-predator behaviour, discuss the implications of these mechanisms in terms of ecology in the aquatic environment, and how they could affect adaptation to environmental changes.

Study Species

The Prey: The great pond snail, Lymnaea stagnalis (L.)

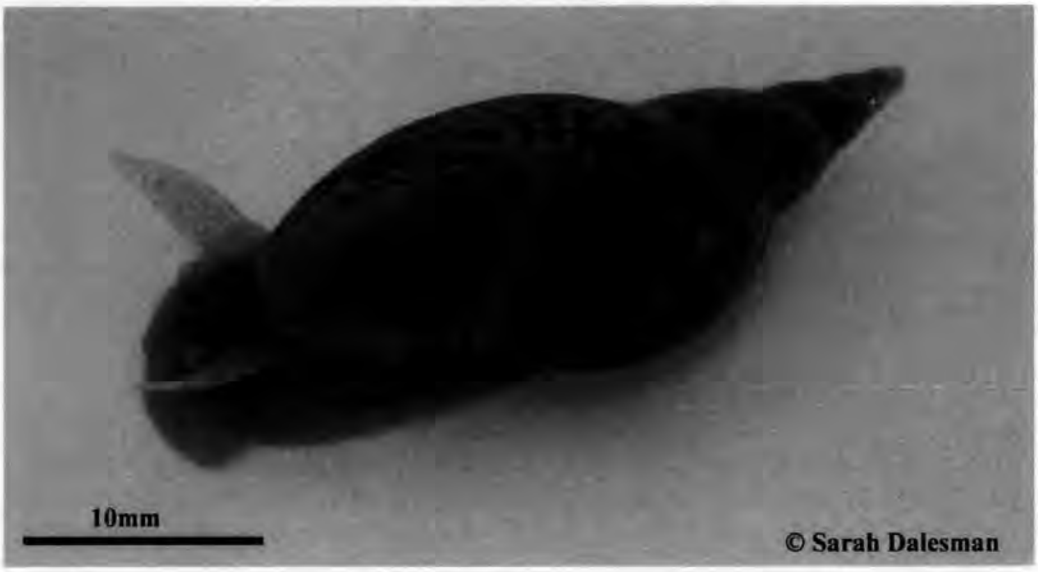

Figure 1.1 **A laboratory reared adult great pond snail, *Lymnaea stagnalis*.**

Lymnaea stagnalis is a common freshwater gastropod, found in drainage ditches, canals, lakes and slow flowing rivers throughout England (Kerney 1999). As a pulmonate this species is 'air breathing', moving to the water surface to take air into the mantle cavity. Oxygen can also be absorbed across the cuticular membrane, particularly via the triangular tentacles which have a large number of blood veins to absorb oxygen. Being air breathing means that they are able to easily move above the water surface, but do risk desiccation after prolonged periods of exposure. *Lymnaea stagnalis* eyes are well adapted for a semi-aquatic lifestyle and can see equally well above and below water (Gal *et al.* 2004).

Lymnaea stagnalis has an average life span of around 2 years (McDonald 1969, Dillon 2000), unlike many of the freshwater pulmonates that are semelparous and only live through a single breeding season (Reavell 1980). Adults are simultaneous hermaphrodites, and normally mature at around 3 months old at temperatures of 15-

20°C, reaching 25-55mm spire height at maturity. Egg masses contain 50-120 eggs (Nichols *et al.* 1971), and a single snail can lay one egg string every 2-4 days when reproductively active (Noland and Carriker 1946, Brown 1979). Under natural conditions breeding occurs between April and October, during which time snails are active, but during winter months they bury in the muddy bottoms and go into a period of torpor (Boycott 1936). In laboratory conditions (12:12 light:dark cycle), however, *L. stagnalis* will produce eggs all year round (McDonald 1969). Juvenile snails hatch out at about 1.3mm long (Noland and Carriker 1946, Dillon 2000), and growth of *L. stagnalis* occurs between 11°C and 28.2°C, with the lowest mortality occurring between 15.7°C and 20.1°C (Vaughn 1953). The optimum constant temperature for hatching eggs and growth of young *L. stagnalis* was found to be 20°C (Vaughn 1944, cited in: McDonald 1969).

Abiotic conditions can affect the growth and survival of *L. stagnalis*. Calcium is commonly thought to be one of the most important environmental requirements for *L. stagnalis*, which is classified amongst 'hard water' species of freshwater gastropods (Macan 1977). The distribution of *L. stagnalis* was found to relate to calcium availability, it is not found in less than 20mg/l (Boycott 1936), and orientates towards calcium rich environments in a choice chamber (Piggott and Dussart 1995). *Lymnaea stagnalis* tends to be more reliant on environmental calcium than other species such as *Radix balthica*, a soft water species, which may acquire the majority of its calcium requirements from food sources rather than environmental (i.e. in dilution in the water) calcium (Piggott and Dussart 1995). In soft water, towards the lower end of its environmental requirements, it is likely that *L. stagnalis* is physiologically stressed (Piggott and Dussart 1995). Although it may persist under such conditions, growth and reproductive output may be diminished. Temperature can also impact on the survival and fecundity of *L. stagnalis* (Dillon 2000), high temperatures (i.e. >30°C) result in mortality, as does freezing, though there may be some local adaptation; populations from small water bodies that can experience higher summer temperatures are better at coping with increased temperature regime in the laboratory (Brown 1979, Janse et al. 1988). In general, temperatures up to 20°C increase the growth rate and reproductive output in *L. stagnalis*, at greater temperatures both growth and reproduction start to decrease (Brown 1979).

There is little evidence for competition between freshwater gastropod species (Brönmark et al. 1991), with abiotic factors, food availability and predator presence considered to be the main limiting factors on distribution and abundance (Boycott 1936, McDonald 1969, Brönmark 1988, Dillon 2000). Density of gastropods is positively related to the abundance and diversity of macrophytes (Brönmark 1988), though this may be related to substrate availability as well as food source. The natural diet of *L. stagnalis* includes diatoms and detritus (Reavell 1980) as well as encrusting algae and macrophytes, and on occasion has been found to include carrion (McDonald 1969). Despite this apparently varied diet, *L. stagnalis* can be raised successfully through to 20 generations in the laboratory on lettuce alone (McDonald 1969).

Freshwater aquatic pulmonates have a large number of potential predators; birds and mammals will consume gastropods at or near the surface (Boycott 1936, McDonald 1969), but they are also consumed by a large number of other aquatic organisms including Osteichthyes, Coleoptera, Hirudinea , Hemiptera, Trichopteran larvae and Dipteran larvae (Hyman 1967, McDonald 1969, Gilinsky 1984, Brönmark 1988). In the aquatic environment the ability to detect potential predators via vision is diminished, so many species use chemosensory signals to detect predation threat (Dodson et al. 1994).

Lymnaea stagnalis has good chemosensory ability (McDonald 1969, Croll 1983) and, as such, it is thought that it will rely on this to detect aquatic predators. They can use both behavioural and morphological defences to avoid predation (Rundle and Brönmark 2001, Chapter 2: Dalesman et al. 2006), though the defence method used will vary depending on the nature of the threat. In the field morphology may differ significantly between populations of *L. stagnalis*, a difference which disappears under laboratory conditions (Arthur 1982), indicating that differences between populations are due to induced changes in growth (Rundle *et al.* 2004). In aquatic gastropods predator induced crawl out response may result in high mortality due to desiccation (Turner *et al.* 2006), and morphological defences can also be costly in terms of growth reduction (DeWitt 1998).

The Predator: tench, Tinca tinca (L.)

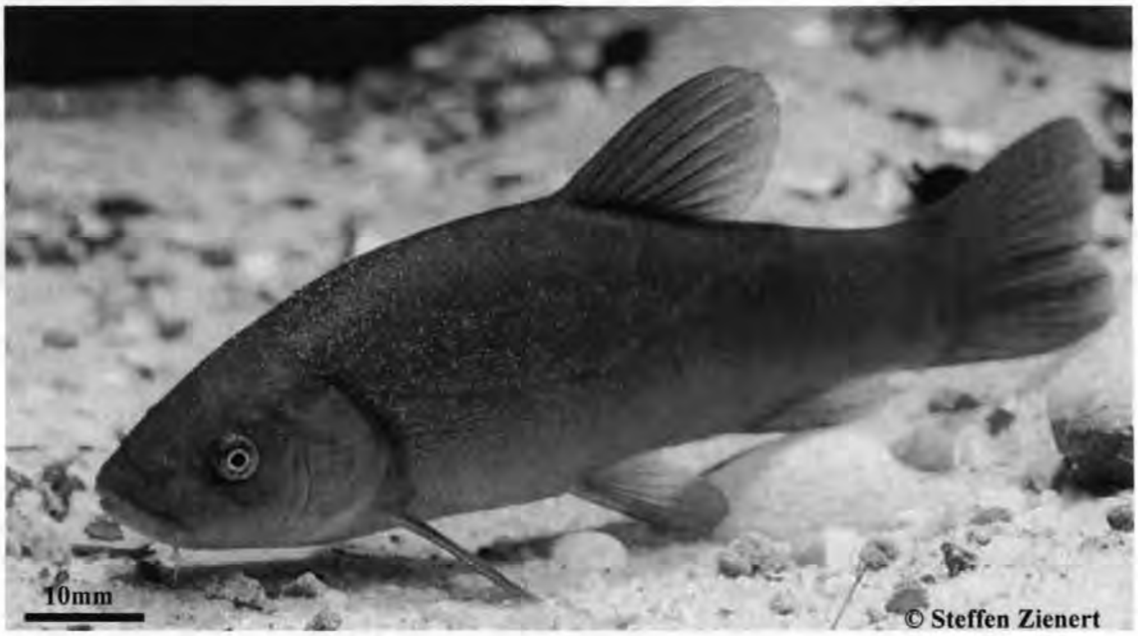

Figure 1.2: A tench, *Tinca tinca.*

Tench, *Tinca tinca*, is a temperate Cyprinid freshwater fish, commonly found in slow-flowing and stagnant water bodies (Fitter and Manuel 1986). They are active from spring to autumn, breeding between May and July in the U.K. (Maitland and Campbell 1992), and during the winter they bury into the mud in the bottom of rivers and lakes, remaining inactive until spring (Altindag *et al.* 1998). Tench are omnivorous, when hunting they are benthic predators feeding on invertebrates and have barbels present on the underside of their jaw to assist with finding prey (Fitter and Manuel 1986). Tench posses pharyngeal teeth, adapted for consuming hard bodied organisms (Weatherley 1959). Gut content analysis of wild caught tench showed that molluscs were not eaten until individuals reached 6.1-8cm, and the number eaten then steadily increased with fish size (Weatherley 1959). For fish in the 11.7-20.2 cm range, pulmonate molluscs were found in the majority of stomach contents analysed and therefore were thought to be the preferred food source when available. Evidence of the impact of tench on benthic invertebrate populations suggest that they can significantly reduce the number of molluscs in an area, but have no significant effect on the non-molluscan invertebrates (Brönmark 1988, 1994, Beklioglu and Moss 1998). This molluscivorous diet made them an ideal species to utilise as a predatory threat for *L. stagnalis* as they will feed readily on this species, and may severely impact on the snail population as they overlap in their distributions. I predicted that *L. stagnalis* would have adapted to respond to reduce the impact of tench predation in their life history, morphology and behaviour.

In the cue production 10±1mm fish were used to provide predator cues. This size of fish feed readily on molluscs (Weatherley 1959), and has a gape size that will allow them to consume the 6mm snails utilised for the behavioural trials (Osenberg and Mittelbach 1989, Shelton et al. 1995). Three fish were placed in 4 l of water for an hour. This length of time was chosen as preliminary trials indicated that there was no effect of cue production time on crawl-out behaviour between 5 minutes and 24 hours (Appendix 1).

Field Sites

Overview

The field sites used to source gastropod populations for rearing in the laboratory were Exeter canal, Exeter, U.K. (50.69N 3.50W), seven canals/ditches on the Somerset levels, an extensive wet meadow system in the Southwest U.K. (grid refernces in Table 1.1), Clyst St Mary (50.71N 3.46W) where *Galba truncatula* were collected and Bodmin Moor (50.56N 4.67W) where *Omphiscola glabra* were collected. The Exeter canal population was used for the first study to investigate the effects of learning on anti-predator behaviour (Chapter 2: Dalesman et al. 2006). However this field site did not allow the environmental comparisons between populations required for the rest of the studies presented in this thesis, and so was not used for further experiments. For this reason the abiotic environmental data collected for the sites on the Somerset levels were not collected for the Exeter canal site. Exeter canal is very similar in size to South Drain, and contained a similar assemblage of gastropod species (pers. obs.) as well as predatory fish (including tench). A comparison of the crawl-out behavioural response to tench and conspecific alarm cues between the Exeter canal population (Chapter 2: Dalesman et al. 2006) and the South Drain population (Chapter 3: Dalesman et al. 2007b) indicates that these populations experiencing similar communities of predators and heterospecific gastropods behave in a very similar way in response to tench predation cues.

Gastropod community

The gastropod species present at the seven different sites on the Somerset levels were identified by qualitative sampling between March 2005 and September 2006 (Table 1.1). Presence/abundance data utilised to test the relationship between assemblage and environment were collected using semi-quantitative sampling (along with abiotic environmental data) on four separate dates during the field season in 2006 (7th April, 22nd May, 18th July and 21st September), with the exception of North Moor small drain, for which species abundance data were only collected on the first three sampling trips. Gastropod presence and abundance was assessed by sampling a 10m stretch of bank by vigorous sweep netting for 10 minutes. The efficacy of this method was confirmed by continuing to net for a further 5 minutes at the same location during the first sampling session, which yielded very few further individuals and no new species. All material collected was sorted in the field and any gastropods present were identified and counted. Where identification could not be made accurately in the field snails were brought back to the laboratory for dissection to confirm the species. The species present at each site differed consistently over the sampling period (Table 1.1).

Table 1.1 Location of sites used in this study on the Somerset Levels and gastropod species present at each site*.

Site	Location	Predatory fish	Species present
South Drain	51.18N 2.88W	Yes	*L.s., R.a., R.b., P.a., A.f., A.v., P.c., P.ca., V.v., B.t.*
Sowy River	51.07N 2.88W	Yes	*L.s., R.a., R.b., P.a., A.f., A.v., P.ca., V.v., B.t.*
N. Moor Main Drain	51.07N 2.96W	Yes	*L.s., L.f., R.b., P.f., A.v., P.c., P.ca., B.t.*
Chilton	51.19N 2.88W	No	*L.s., L.f., R.b., A.v., P.c., P.p., P.ca., B.t.*
Little Hook	51.06N 2.87W	No	*L.s., L.f., R.b., A.v., P.c., P.p., P.ca., B.t.*
Wistaria	51.07N 2.98W	No	*L.s., L.f., R.b., A.v., P.c., P.ca., B.t.*
N. Moor small	51.08N 2.96W	No	*L.s., L.f., R.b., A.v., P.c., P.ca., B.t.*

* Species code: *L.s.* (*Lymnaea stagnalis*), *L.f. (Lymnaea fusca), R.a. (Radix auricularia), R.b. (Radix balthica), P.f. (Physa fontinalis), P.a. (Physella acuta), A.f. (Ancylus fluviatilis), A.v. (Anisus vortex), P.c. (Planorbarius corneus), P.p. (Planorbis planorbis), P.ca. (Planorbis carinatus), V.v. (Viviparus viviparus), B.t. (Bithynia tentaculata).*

N.B. *Omphiscola glabra* and *Galba truncatula*, used to provide cues in Chapter 6 were never found at the sampling sites listed and were collected at Bodmin Moor (50.56N 4.67W) and Clyst St. Mary (50.71N 3.46W) respectively.

Fish community

The presence of predatory fish, including tench, at three of the sites (South Drain, Sowy River and North Moor Main Drain) was confirmed using data from the Taunton Angling Association (pers. com.). Electro-fishing using 240vDV equipment (Severnside Industrial, U.K.) was carried out on 14th June 2006 to confirm the absence of predatory fish in the small ditches (Chilton, Little Hook, Wistaria and North Moor small drain). Permission for electro-fishing was obtained from the Environment Agency (licence numbers: B/NW/090506/E20 to B/NW/090506/E23, B/NW/180506/R1) and the landowners before sampling was carried out. Tench actively feed during June (Billard and Monod 1997), and also reproduce at this time of year (Breder and Rosen 1966), so there is potential for both adult and larval fish to be present. A minimum of a 20m stretch of each site was sampled for 30 minutes. The only fish species present at all four sites was the nine spined stickleback (*Pungitius pungitius* L.). This species eats small planktonic and benthic invertebrates, but has not been found to feed on molluscs (Scott and Crossman 1973, Maksimenkov and Tokranov 1995, Thiel et al. 1996).

Environmental data

Environmental data from the Somerset levels were collected from each of the sites on four dates during the field season in 2006 (7th April, 22nd May, 18th July and 21st September), with the exception of North moor small drain, for which environmental data were only collected on the first three sampling trips. Width and depth were estimated at each site (mean of 5 measurements). Temperature (°C), conductivity (μS adjusted for temperature), salinity (p.p.t.) and dissolved O_2 (mg/l and % saturation) were measured on site using Solomat 520 C probe (Zellweger Analytics, Poole, U.K.). Two water samples were taken from each site in acid washed polypropylene bottles for analysis of nutrient and metal ions. pH of each water sample was measured in the laboratory using a HI 9023 microcomputer (Hanna Instruments Ltd., U.K.). Nitric acid (at 1% concentration) was then added to the water samples taken to assess metal ion content to ensure the metal ions remained in solution, and samples were frozen on the day of collection until they could be analysed. Nitrate and phosphate concentration (mg/l) were analysed using a Dionex autoanalyser (Camberley, U.K.), $[Ca^{2+}]$ and $[Mg^{2+}]$ using atomic absorption spectroscopy (Varian SpectrAA 50B: Varian Inc. USA). The mean, minimum and maximum values for all the environmental data are presented separately for sites containing predatory fish and sites lacking predatory fish (Table 1.2).

Table 1.2 Environmental Data: maximum, mean and minimum values for environmental variables measured. Data shown are separated between sites containing predatory fish (n = 3) and sites not containing predatory fish (n = 4).

Variable measured	Predatory fish present			No predatory fish		
	maximum	mean	minimum	maximum	mean	minimum
[Mg^{2+}] mg/l	11.80	6.77	2.90	27.20	9.89	3.20
[Ca^{2+}] mg/l	147.6	76.7	35.4	184.6	86.7	23.7
Nitrate mg/l	10.83	4.13	0.14	1038.60	135.20	0.10
Phosphate mg/l	0.764	0.296	0.032	0.864	0.143	<0.001
Width (m)	22.00	14.70	6.00	2.90	2.10	1.80
Depth (cm)	130.00	103.33	70.00	70.00	56.67	45.00
pH	8.80	7.90	7.45	8.20	7.45	7.10
%O_2	101.30	49.59	2.20	58.80	30.08	3.90
O_2 mg/l	11.14	5.02	0.19	6.30	3.08	0.380
uS (adj. for temp.)	849.0	704.4	564.0	1170.0	828.8	650.0
Temperature (°C)	25.70	16.74	10.10	27.40	15.08	7.20
Salinity p.p.t.	0.410	0.352	0.300	0.600	0.410	0.300

Assemblage analysis

Interrelationships between sites in terms of their snail assemblages and physiochemistry were explored using multivariate data analysis performed using PRIMER v.5 (PRIMER-E Ltd. Plymouth Marine Laboratory. U.K.). Similarities of the gastropod assemblage between sites were analysed using fish (presence/absence), date of collection and field site as factors. Due to the large amount of variability in abundance data (from 1 to >500 individuals) data were fourth root transformed, the most robust transformation found to downweight the effects of very abundant species (Clarke and Warwick 2001). A Bray-Curtis (Bray and Curtis 1957) similarity matrix (on percentage differences) was calculated on the abundance/presence data. Multi-dimensional scaling (MDS: Kruskal 1964, Clarke and Warwick 2001) was used to calculate the relative position of each data point for visual interpretation of the data. Dispersion was calculated using multivariate dispersion indices (MVDISP: Warwick and Clarke 1993) to give a comparison of how dispersed the data are between site with and without predatory fish. Analysis of similarities (ANOSIM: Clarke and Green 1988) was used to test for similarities between assemblages based on the factors date of collection, site of collection and presence/absence of predatory fish. A Kruskal stress value of 0.17 (Kruskal 1964) was calculated for the positioning of these points by MDS, suggesting that the relative positioning of the points calculated can be viewed with confidence. The gastropod assemblage differed significantly between sites (Figure 1: ANOSIM: R = 0.404, P = 0.001: Appendix 2: Table A2.1), and also differed significantly depending on whether predatory fish are present at the site (Figure 1.3: ANOSIM: R = 0.185, P = 0.003), but there was no signifcant effect of sampling date. The dispersion of the data was greater at sites without predatory fish (1.128) than at sites containing predatory fish (0.797: Figure 1.3).

The greater dispersion of assemblage at the sites lacking predatory fish indicates that these sites differ to a greater extent in their environment both between sites and also temporally (Figure 1.3). This is particularly evident at the Chilton and North Moor small drain sites, both of which have a greater within site dispersion in their assemblages than is found between the sites containing predatory fish. This may be due to high disturbance levels at these sites as small drains are cleared sporadically removing all vegetation and deepening the water channel, whereas the larger sites do not undergo such broad scale disturbance.

The effect of environmental variables on gastropod assemblages was also calculated. Variables measured for comparison were: [Mg^{2+}], [Ca^{2+}], nitrate, phosphate, pH, O_2 mg/l, μS (adj. for temperature), temperature (°C), p.p.t., channel width and channel depth. Spearman Rank Correlation was used to compare biotic (assemblage) and environmental variables (BIO-ENV: Clarke and Ainsworth 1993). Many of the environmental variables were highly intercorrelated, apart from nitrate concentration which was not correlated with any other environmental variables (Appendix 2: Table A2.2). Magnesium concentration (mg/l) had the highest correlative value with assemblage, with pH explaining the next greatest degree of variation (Appendix 2: Table A2.3). Temperature also explained about a quarter of the variation between assemblages, though this was discounted from the final combination due to strong intercorrelation between temperature and Magnesium concentration (Appendix 2: Table A2.2). Of the environmental variables, [Mg^{2+}] and pH provided the best combination of variables, not confounded by intercorrelations, to explain the variation between assemblages ($t = 2.555$, $P = 0.017$, $n = 25$).

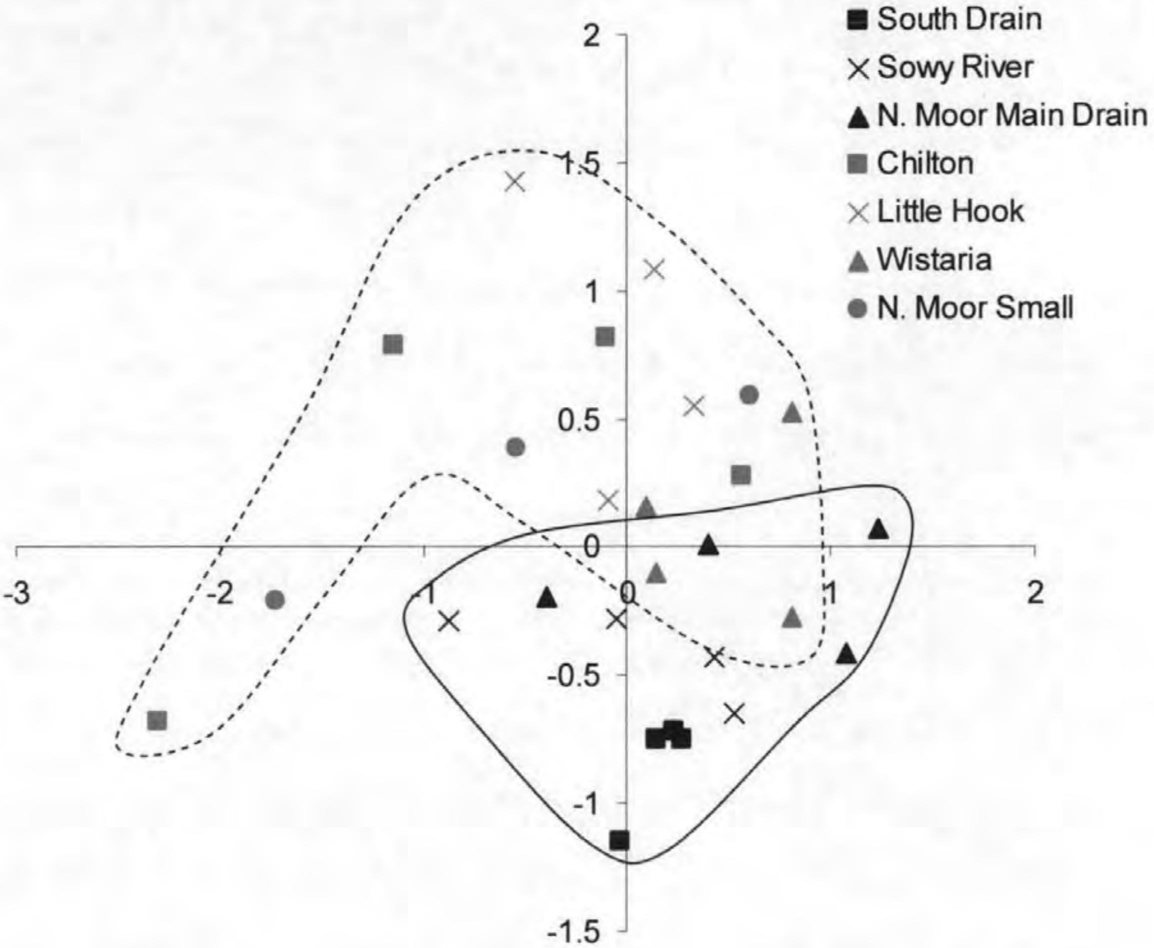

Figure 1.3: Multi-dimensional scaling (MDS) of assemblage data (stress value = 0.17). Sites are shown as either fish (black symbols) or no fish (red symbols), with the same shaped symbol indicating that two sites are found in close geographic proximity (i.e. the two sites labelled with squares, South Drain and Chilton, are in closer proximity to one another that any other sites) with the exception of N. Moor small drain which is as close to North Moor Main Drain as Wistaria and has no predatory fish. Clustering of fish sites (solid line) and no-fish sites (dashed line) is indicated.

The variation in gastropod assemblages between sites containing predatory fish and those not containing predatory fish may be due to overall differences in other environmental variables as well as the direct effects of fish predation. Fish predation has been found to have a significant influence on the abundance of gastropods, with tench predation dramatically reducing the abundance of pulmonates (Brönmark 1988, 1994). However abiotic factors can also influence the presence of gastropods, indeed the calcium concentration is thought to be a major factor dictating the distribution of aquatic gastropods, with calciphiles such as *Lymnaea stagnalis* absent where [Ca^{2+}] drops below 20 mgl^{-1} (Boycott 1936). The general cleanliness of the water, such as the extent of eutrophication can also significantly affect gastropod distributions (Boycott 1936). In this study [Mg^{2+}] and pH were found to be good indicators of assemblage, both these variables showed strong intercorrelations with other environmental variables (Appendix 2: Table A2.2). The pH was higher at the sites containing predatory fish ($t = 3.54$ $P = 0.002$ $DF = 20$), but the [Mg^{2+}] didn't differ significantly between sites with and without predatory fish. This suggests that [Mg^{2+}] is acting independently on the gastropod assemblage, potentially as a general indicator of water hardness (Baird 1995).

The lack of effect of environmental calcium may be due to the fact that calcium has a limiting effect on calciphilic gastropod assemblage below 20mg/l (Boycott 1936). From the field data the minimum calcium concentration found at any sites in this study was 23mg/l, with fluctuations to considerably higher levels during most of the year (Table 1.2). Whilst levels this low may affect shell growth (Rundle *et al.* 2004), it is not going to affect the assemblage found. Indeed the ubiquitous presence of hardwater species such as *Lymnaea stagnalis*, *Bithynia tentaculata* and *Anisus vortex* (Macan 1977) at all the sites acts as an indicator that calcium is not limiting on the Somerset levels.

Animal Husbandry

A full list of the gastropod species used during this study and the locations where they were caught are shown in this chapter (Table 1.1) and Chapter 6 (Table 6.1). Husbandry techniques were similar in the majority of cases, and variations in husbandry techniques are explained below. Wild collected adults of each species used (except *Galba truncatula*, see below) were collected by sweep netting in the macrophyte vegetation at the collection sites then returned to the laboratory in containers with damp vegetation to retain humidity. *Galba truncatula* was collected by picking individual adults by hand from the mud and vegetation surface around indents caused by cow trampling. Adults were retained in single species groups in plastic aquaria in 4 l of aerated artificial pond water (ASTM 1980) as tap water may contain copper ions which may be toxic to *L. stagnalis*, even when strongly diluted (Noland and Carriker 1946). Aeration was used as it has been found to increase egg laying rate (Brown 1979), so potentially decreases physiological stress. Snails were held at 15°C in a 12:12 light:dark cycle, except *G. truncatula* and *L. stagnalis* for the work presented in chapters 4 & 5 (see below). It was found that *G. truncatula* did not survive or reproduce well at 15°C, and so this species was held in a separate laboratory at 20°C where growth, survival and reproduction were greatly improved; all other variables were kept the same as the other species held at 15°C. *Lymnaea stagnalis* was maintained at 15°C to produce F1 generation juveniles to carry out behavioural trials for chapters 2, 3 and 6, however due to the need to reduce generation time for selection experiments they were maintained at 20°C for the studies in chapters 4 & 5.

The density at which adults were held in aquaria depended on the species as some gastropod species do very poorly at high density, with reduced growth, survival and reproduction (Noland and Carriker 1946, McDonald 1969). Fast growing species that may go through more than one generation in a season, such as *Radix balthica*, *Lymnaea fusca*, *Omphiscola glabra*, *Bithynia tentaculata*, *Planorbis planorbis* and the physids tended to survive well and reproduce quickly at high density, and as many as fifty adult snails may be held in a 4 l tank. Slower growing species which only go through one generation a year under natural conditions, such as *Radix auricularia*, *Planorbarius corneus* and *Lymnaea stagnalis* survived and reproduced better at lower densities of eight to ten adults per 4 l of water (McDonald 1969, Brown 1979, Janse et al. 1988). Adults lay egg strings, which vary in size depending on the species used, the exact rate of production was only measured qualitatively in *L. stagnalis* using casual observations. Following egg laying, adults were transferred to a new tank. This reduced the stress on juvenile snails by reducing adult waste products, so improving water quality (McDonald 1969) and reduced juvenile mortality. It also reduced the potential for the transfer of parasites from adults to hatchlings, which was particularly important in the case of *Lymnaea stagnalis* as parasitic infection has been shown to affect the anti-predator behaviour in another pulmonate snail, *Physa integra* (Bernot 2003). Other techniques used to reduce the possibility of parasite transmission included sterilising cleaning nets between cleaning each tank, and using separate nets for laboratory reared and wild caught snails.

All snails used for both behavioural trials and alarm cue production were at least F1 generation laboratory reared individuals, and on occasion F2 or F3 generation. This demand for laboratory-reared snails meant that conditions had to be maintained in such a way as to maximise reproductive potential. As well as the issues of density and water cleanliness, calcium and food availability were also considered. Egg production in *Radix balthica* is promoted by feeding spinach (Dillon 2000), possibly due to the relatively high calcium content of this leaf, so both iceburg lettuce and spinach were fed to the laboratory populations. Food was fed *ad libitum* as over-crowding on food has been found to reduce growth (McDonald 1969). The selection for spinach versus lettuce differed between snail species and also between juveniles and adults, though both were eaten to some extent in all cases. Though a pattern was not noted in the consumption it has been previously found that calciphiles such as *L. stagnalis* rely more heavily on environmental calcium, whereas soft water species such as *Radix balthica* gain a large proportion of their calcium from food sources (Piggott and Dussart 1995), so it is possible that this factor alters the food preferences of different species.

Calcium can affect both the adult growth rate (energy budget) and also egg production. For example, egg production by the pulmonate snail, *Biomphalaria glabrata*, rose with environmental calcium availability from practically no egg production in [Ca^{2+}] 0mg/l, rising steadily to a maximum at 80 mg/l (Thomas *et al.* 1974). Although hard water species such as *L. stagnalis* may be found in environments with [Ca^{2+}] levels above 20mg/l (Boycott 1936), availability has also been shown to affect the growth potential of *L. stagnalis* (Rundle *et al.* 2004), so it was provided at a level of 90mg/l in the water in both holding tanks and during behavioural trials. This level is about half the maximum concentration experienced by the snail populations on the Somerset levels (184.6 mg/l), but considerably higher than the minimum experienced (23.7 mg/l) and is close to the average [Ca^{2+}] they experience of 75-90 mg/l (Table 1.2).

Thesis Aims

In this thesis I investigate the mechanisms that govern the way an animal responds behaviourally to the threat of predation. Many studies demonstrate that animals from different populations differ in the way they respond to predation, or differ in morphology between populations which is presumed in part to be due to predator presence. There is, however, still relatively little known about whether these responses have a fixed genetic basis or whether the animal has evolved induced defences that respond to current environmental conditions. I used the gastropod *Lymnaea stagnalis* as a model organism to investigate these questions. This is a relatively easy species to obtain and rear in the laboratory in large numbers that has a simple set of possible behavioural responses to predation cues, allowing relatively complex questions about the behavioural mechanisms to be tested in a simple experimental set up. The specific questions addressed in this thesis are detailed in the following chapters (2-6) and brief outlines are provided below:

Chapter 2. Here I addressed firstly whether juvenile *L. stagnalis* demonstrate an innate response to cues associated with predation threat and then asked whether recent experience of cues associated with a predation event can alter this innate response. An actively foraging predator was predicted to represent more of a risk to *L. stagnalis* than one not foraging, so it was predicted that the innate response to predator cues would be intensified following experience of predation cues.

Chapter 3. In this chapter I asked whether the innate responses found in Chapter 2 varies between populations depending on the local predator regime. I considered it necessary to account for population variation in this study as different populations are exposed to different predators and so may exhibit local adaptation to their environment, therefore, results obtained from one population could not be generalised across the species as a whole. An investigation of innate differences between populations would also give insight into whether local adaptation had occurred.

Chapter 4. The general aim of this chapter was to investigate how rapidly a population might respond to altering predator regimes. I firstly address whether the population differences found in chapter 3 were due to local genetic adaptation for a fixed innate response or transgenerational induction. Secondly, I tested how rapidly the differences seen between wild caught populations in chapter 3 could be selected for.

Chapter 5. In this chapter I ask whether plasticity in behaviour occurred alongside fixed genetic responses. I also investigated whether induced responses were inherited along with the mean of an innate behaviour, or if they act independently, and discuss the potential implications for species invasions or colonisation on recognition of a predator.

Chapter 6. Here I investigated the relationship between *Lymnaea stagnalis* and other gastropod prey, addressing the questions of whether *L. stagnalis* is able to use alarm cues from prey guild members to assess predation risk, whether the recognition of alarm cues has a phylogenetic and ecological basis, and if there are potential implications of local adaptation.

CHAPTER 2

Cue association and anti-predator behaviour in a pulmonate snail, *Lymnaea stagnalis*.

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Abstract

Associative learning may help to offset costs of unnecessary escape behaviour by providing accurate information about the current risk to potential prey. Here I demonstrate cue association learning in naïve gastropods. Juvenile laboratory reared great pond snails, *Lymnaea stagnalis* (L.) showed an innate ability to respond with anti-predator behaviour to odour from a natural predator, tench, *Tinca tinca* (L.). The main anti-predator behaviour of *L. stagnalis* to a high perceived predation risk from *T. tinca* was to crawl above the water line (crawl-out response). The crawl-out response of snails was significantly increased in the presence of fish cues, but maximum response occurred when alarm cues (crushed conspecifics) were also present. However, a second experiment demonstrated that, following pre-exposure to tench odour and alarm cues, responses to tench cue alone were similar to those seen in response to tench plus alarm cues presented together during the first experiment. Hence, *L. stagnalis* are apparently capable of relating potential predation risk to recent experience. A final experiment showed that this raised level of avoidance persisted for at least eight days, suggesting that this behaviour may be retained over time scales relevant to predation risk in the natural environment. The ability of organisms to modify anti-predator behaviour based on recent experience, as found in *L. stagnalis*, would allow costs associated with unnecessary response to be reduced whilst effectively avoiding active predators.

Introduction

Failure to respond to a potential predator may be fatal, however unnecessary escape behaviour may have direct energetic costs as well as costs associated with reduced opportunity to feed or reproduce (Lima and Dill 1990). Sensory information obtained about a predator may assist an organism in assessing the potential risk accurately and therefore reduce these costs (Lima and Dill 1990, Chivers and Smith 1998). A range of sensory inputs can be used in predator detection; however, where other cues are impaired by environmental or physiological constraints, for instance visual cues in an aquatic system, chemical cues may provide an alternative (Dodson et al. 1994). Predator released kairomones (Dicke and Sabelis 1988) can give information about the presence, proximity, physiological state and diet of potential predators (Kats and Dill 1998). Injury released chemical cues from conspecifics frequently induce an anti-predator response in aquatic taxa (Wisenden 2000). However, although a good indication of potential threat in isolation, these types of cue may not provide accurate information about the nature of current risks, and so may elicit inappropriate anti-predator responses.

Several studies have demonstrated that combining alarm cues produced by injured conspecifics with predator kairomones elicits the greatest anti-predator response in organisms, suggesting that this represents the greatest perceived risk. This combination of cues either takes the form of the predator feeding on conspecifics (Crowl and Covich 1990, Loose and Dawidowicz 1994, Sih and McCarthy 2002, Turner and Montgomery 2003, Jacobsen and Stabell 2004) or predator cues paired with crushed conspecific, thought to simulate a predation event (Turner 1997, DeWitt et al. 1999, Rundle and Brönmark 2001, Cotton et al. 2004). Some prey organisms that respond to predator cues paired with conspecific cues have been found to lack response to predator cues alone (Turner 1996, Jacobsen and Stabell 2004). An important question is whether this is due to either an inability to recognise the predator, or due to a lower perceived risk from a non-feeding predator. The ability of gastropods to differentiate between the types of predator present from cues paired with injured conspecific suggests that the latter may be the case in some predator-prey systems. For instance, the freshwater snails *Physella gyrina* and *P. integra* are able to differentiate between cues from different predators, crayfish, *Orconectus rusticus* and pumpkinseed fish, *Lepomis gibbosus*, when presented with crushed conspecific snail, and respond with appropriate anti-predator behaviour (Turner et al. 1999, Bernot and Turner 2001).

Experience of predation cues has been demonstrated to be an important element in the development of anti-predator behaviour in a wide range of vertebrates as well as invertebrates. Learning from prior experience of predation has been found to be integral to most fish species' ability to recognise predators (Brown 2003, Kelley and Magurran 2003). For instance, fathead minnows, *Pimephales promales*, learn to recognise a potential predator, northern pike, *Esox lucius*, from chemical cues produced by pike feeding on minnows (Mirza and Chivers 2001b). Learning about predators through diet information, or pairing of predator cues with damaged conspecifics, has also been noted in several invertebrates including: damselflies, *Enallagma* spp. (Chivers et al. 1996), crayfish (Hazlett et al. 2002), whelks, *Buccinum undatum* (Rochette et al. 1998) and an aquatic flatworm, *Dugesia dorotocephala* (Wisenden and Millard 2001). In these cases it was unclear as to whether the prey species were learning to identify a potential predator *per se*, or whether the association between cues was providing information about the perceived risk from the predator.

The great pond snail, *Lymnaea stagnalis* (L.), is slow moving relative to many molluscivorous predators, and lives in an aquatic environment where visual information may often be hindered by turbidity or dense vegetation. Prior studies have shown that *L. stagnalis* responds to chemical cues from potential predation risk by either sheltering in crevices or crawling above the water line (Snyder 1967, Rundle and Brönmark 2001). As such it is predicted that *L. stagnalis* will use chemical cues as an important source of information about predators (Wisenden 2000), which makes it an ideal model organism to study the way in which chemical cues can be used to assess predation risk. The aims of this study were to use naïve lab-reared snails to: (1) determine the extent of innate responses to predator cues, (2) assess the ability of *L. stagnalis* to form cue associations, (3) determine the effect of these associations on behavioural responses, and (4) determine the longevity of these associations. I predicted that: (1) *L. stagnalis* would respond to higher risk by displaying a stronger anti-predator response, (2) the perceived risk from predator cues would be enhanced by cue association with a simulated predation event, and (3) perceived risk would diminish over time since exposure to a simulated predation event.

Methods

Study Organisms

Adult *L. stagnalis* were collected from Exeter canal, U.K. (50° 69' N 03° 50' W) in April 2004. In the laboratory adults were kept in 6 l Savic plastic aquaria (Aquatics online, U.K.) in 4 l of aerated artificial pond water (ASTM 1980) with 90mgl^{-1} [Ca^{2+}] (Rundle et al. 2004). They were maintained at 15±1 °C under ambient light levels, and fed on Iceberg lettuce. Experimental snails were bred from egg masses laid a minimum of two months post collection, to minimise the potential influence of adults experience on juvenile development and behaviour. Juvenile snails were then maintained separately in the same conditions as the adults. Individuals with a standard spire height of 6±0.5 mm were used for behavioural experiments.

Tench, *Tinca tinca*, were taken from a laboratory stock originally obtained from Emperor Tropicals & Water Garden Centre, Plymouth, U.K. They were maintained in aerated and filtered water at 15±1°C under ambient light levels in 25 l tanks at a stocking density of 12 fish per tank (0.006 kgl^{-1}). Gravel substrate and shelters positioned in the tank were used to mimic natural conditions. The tench were fed Nutri-flake (Hozelock Cyprio, U.K.), which contains no mollusc extracts, and hence avoided potential dietary cues affecting the trials. No experimental manipulations were carried out on these fish and the welfare of the fish was carefully considered throughout. Following cessation of the trials the tench were retained as laboratory stock.

Odour Production

Predator and alarm cues were produced in the same way in all the experiments. *T. tinca* were used to produce predator cue as they are a known natural predator of freshwater gastropods (Brönmark 1994). Predator cue water was produced by placing three *T. tinca* (length 10±1 cm) into 4 l of aerated artificial pond water for 1 hour. Alarm cue was produced by mixing three crushed juvenile *L. stagnalis* (6±0.5 mm) into 4l of aerated artificial pond water. Predator plus alarm cue was produced by mixing three crushed *L. stagnalis* (6±0.5 mm) into 4 l of tench cue water.

Behavioural Assay

Behavioural trials were carried out in the same way for all experiments under laboratory conditions analogous to the conditions in which the snails were maintained. Behavioural chambers consisted of a of a white plastic dish, 165mm diameter x 60mm depth (A.W.Gregory & Co. Ltd., U.K.), with a longitudinally sectioned white PVC pipe, 36mm long, 30mm diameter, attached open side down to the centre using non-toxic sealant to provide a refuge. Juvenile snails were acclimated to behavioural chambers for 24 hours in 630ml of artificial pond water prior to behavioural assays. 70ml of cue water was added to each chamber at the start of the behavioural assay to give a 10% final concentration of cue water. Preliminary trials using predator cue alone demonstrated that this was adequate odour strength to initiate an anti-predator response in *L. stagnalis*.

The position of each snail within the behavioural chambers was recorded every 5 minutes for 2 hours following cue addition. Crawl-out behaviour (where the snail moved above the waterline) was found to be the main anti-predator response of *L. stagnalis* to the predator, with the refuge not used, hence only results from crawl-out behaviour are presented here. Crawl-out behaviour was analysed using two variables: the proportion of total time spent crawled out of the water; and the latency to crawl-out, calculated as the proportion of total time during the trial before crawl-out behaviour was observed (Cotton et al. 2004). Proportion of total time spent crawled out was used in conjunction with the time at crawl-out as snails returned beneath the water line on occasion. The results of a power analysis on our preliminary study indicate that using 20 or more individuals per treatment gives an 80% or higher probability of detecting an effect of treatment.

Risk Level and Avoidance Behaviour

Following acclimation to behavioural chambers, four treatments were used to assess the response of *L. stagnalis* to varying degrees of predation risk: (i) control (untreated artificial pond water), (ii) alarm cues alone (crushed conspecifics), (iii) predator odour alone and (iv) predator odour plus alarm cues. It was predicted that the control would represent the lowest risk level, predator plus alarm cues the highest, with predator or alarm cues presented individually being perceived as an intermediate risk. Individuals were randomly assigned to a treatment level such that equal numbers of snails were exposed to each treatment per trial. Twenty-four snails were used for each treatment combination.

Cue Association

To assess whether a simulated predation event (predator and alarm cues together) increased the perceived risk from a predator, snails were exposed to a conditioning treatment where both cues were presented together, followed by exposure to predator cues alone during the behavioural assay. This would represent experience of a predation event in the environment followed by the predator returning to the environment without subsequent predation occurring.

Juvenile snails were pre-exposed for 48 hours, maintained in 1 l of water in 1.6 l Savic tanks in one of four conditioning treatments: (i) control (untreated artificial pond water), (ii) alarm cues alone (crushed conspecifics), (iii) predator odour alone and (iv) predator odour plus alarm cues. Cue water was produced as in behavioural assays and again added at a concentration of 10% by volume.

Following pre-exposure individual snails were randomly allocated and acclimated to behavioural arenas in control water for 24 hours. Prior to behavioural assays, either control water or predator cue was added to the behavioural arena, so that for each trial half of the snails that had been conditioned to each treatment were exposed to control or predator cue. A total of one hundred and eighty-four snails were used, forty-six were exposed to each conditioning treatment, and then half of each group was exposed to predator odour during behavioural assays and half to control resulting in a total of twenty-three snails for each treatment combination.

Persistence of Cue Association

To assess the persistence of cue association, juvenile snails were pre-exposed as before for 48 hours in either (i) control (artificial pond water only) or (ii) predator odour plus alarm cues. They were then either: (i) acclimated in behavioural arenas for 24 hours, (ii) placed into control water for 3 days and then acclimated for 24 hours or (iii) placed into control water for 7 days then acclimated for 24 hours. This resulted in total periods of 1, 4 and 8 days since cue exposure prior to behavioural assay. Snails were randomly allocated to a behavioural arena, and exposed to either control or predator odour during the behavioural assay. Twenty-four snails were used for each treatment combination.

Data Analysis

Data were analysed as balanced ANOVAs using GMAV5, except in the case of the cue association experiment which was initially analysed using a General Linear Model procedure in MINITAB13 due to an unbalanced design between data collection trials. As no significant effect of trial was found, trial was removed as a factor and further analysis was carried out using a balanced ANOVA in GMAV5. All proportion data were square-root arcsine transformed prior to analysis (Underwood 1997). Student-Newman-Keuls tests (SNK) were used for post-hoc comparisons.

Log likelihood tests (G-tests) were used to assess the number of snails crawling out in response to different treatments, data were N+1 transformed when zeros were present, and adjusted by Williams' correction (Sokal and Rohlf 1995). α was corrected using the Bonferroni correction to $\alpha' < 0.025$ to test cue association and $\alpha' < 0.0125$ to test the persistence of cue association as multiple comparisons were required to analyse the data (Sokal and Rohlf 1995).

Results

Risk Level and Avoidance Behaviour

The number of *L. stagnalis* crawling out differed significantly between treatment levels (G-test: $G_3 = 66.750$, $P < 0.001$): no snails crawled out in either the control or alarm cue treatments; however 15 snails crawled out in response to predator alone, and 21 snails crawled out in response to predator plus alarm cues.

Latency to crawl-out was significantly shorter in response to predator plus alarm cues compared to predator cue alone, indicating a more rapid response with increased risk (Table 2.1; SNK test: $P < 0.05$: Fig. 2.1a). The proportion of time spent crawled out of the water was significantly greater in response to predator cue paired with alarm cue than to predator cue alone (Table 2.1; SNK test: $P < 0.05$: Fig. 2.1b).

Table 2.1 Latency to crawl-out and proportion of total time spent crawled out in response to exposure to tench cues and alarm cues. N = 24 for all treatment combinations

		Latency			Proportion of time		
Source	**DF**	**MS**	**F**	**P**	**MS**	**F**	**P**
Trial (Tr)	2	565. 7	1.53	0.225	701.0	2.49	0.089
Alarm cue (Al)	1	2600.4	6.99	0.010	6511.9	23.17	<0.001
Tench cue (Te)	1	52921.6	142.16	<0.001	31123.6	110.74	<0.001
Tr x Al	2	647.5	1.74	0.182	127.2	0.45	0.638
Tr x Te	2	565.7	1.52	0.225	701.0	2.49	0.089
Al x Te	1	2600.4	6.99	0.010	6511.9	23.17	<0.001
Tr x Al x Te	2	647.5	1.74	0.182	127.2	0.45	0.638
Total	95						

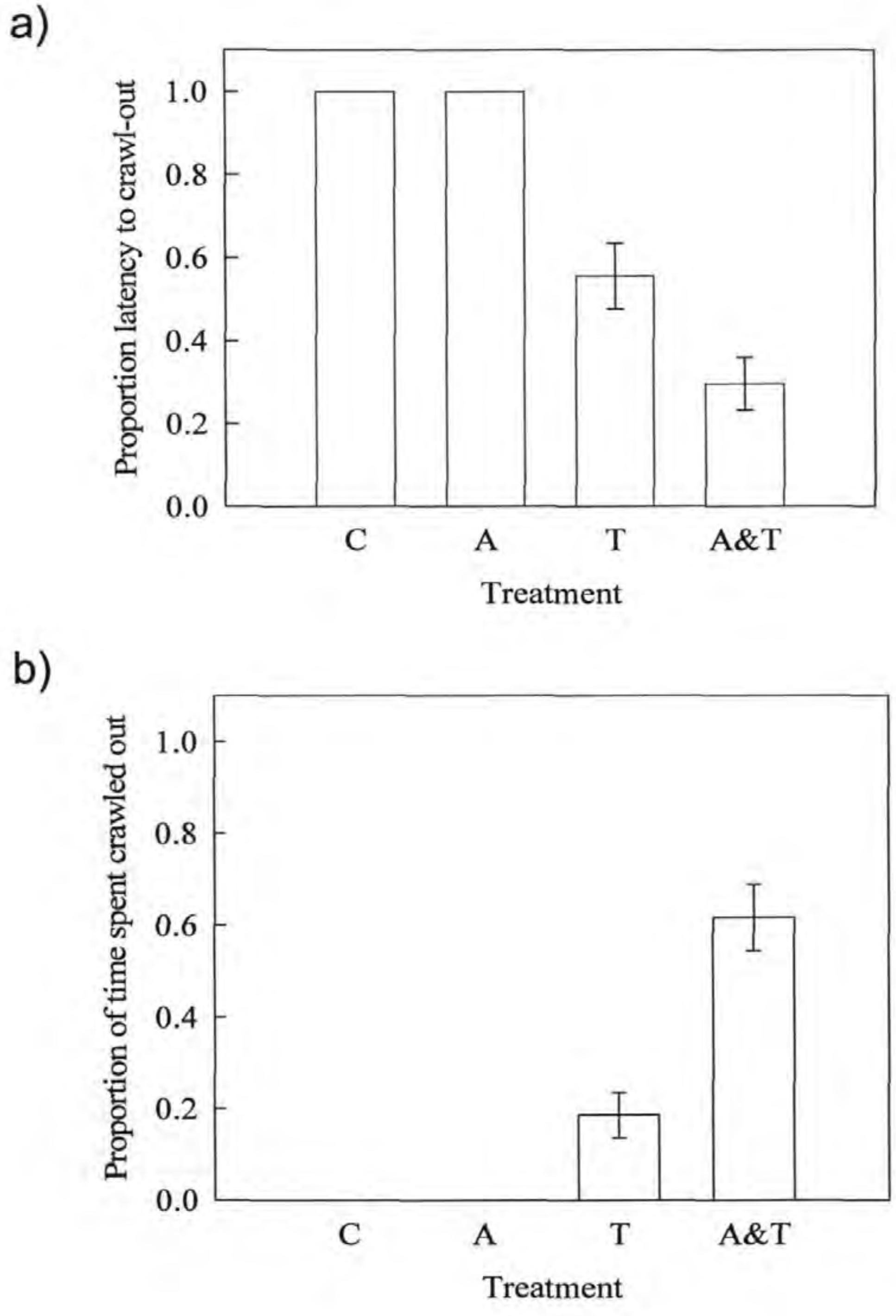

Figure 2.1The mean (± S.E.) proportion; (a) latency to crawl-out and (b) time spent out of the water by *L. stagnalis* in response to different treatments during behavioural assay (duration 2 hours): control (C), alarm cues alone (A), predator odour alone (T) and alarm plus predator cues (A&T)

Cue Association

Cue association was found to significantly affect the response of *L. stagnalis* to predator cues (Fig. 2.2). SNK tests ($P < 0.05$) showed the effect of pre-conditioning to predator plus alarm cue significantly decreased the latency to crawl-out compared to control or either cue presented alone (Table 2.2: Fig. 2.2a). There was a significant effect of trial on latency to crawl-out (Table 2.2). However this was only significant in two pair-wise tests between trials so is not considered to have an overall impact on the results found. The proportion of time spent crawled out was significantly greater in response to predator cue during behavioural assays when *L. stagnalis* had been pre-conditioned to predator paired with alarm cue than when individuals had been pre-conditioned to control or either cue alone (Fig. 2.2b; Table 2.2; SNK test: $P < 0.05$). There was a significant effect of trial, though this was found to be caused by a change in the magnitude of response on only one out of the eight trials on individuals that had been pre-conditioned to alarm cues, so is not considered to have an overall effect on the results (Table 2.2; SNK test: $P < 0.05$).

Overall, significantly more snails crawled out when exposed to predator cue during behavioural assays than those exposed to control conditions during behavioural assays (G-test: $G_1 = 30.974$, $P < 0.001$). The number of snails crawling out in both control and predator cue during behavioural assays was significantly greater when they had been pre-exposed to alarm plus predator cue compared to control or either cue presented alone (Control; G-test: $G_3 = 10.307$, $P = 0.016$: Predator; G-test: $G_3 = 30.324$, $P < 0.001$).

Table 2.2 Latency to crawl-out and proportion of total time spent crawled out in response to exposure to tench cues following previous exposure to tench and/or alarm cues 24 hours prior to behavioural assays. N = 24 for all treatment combinations

		Latency			Proportion of time		
Source	**DF**	**MS**	**F**	**P**	**MS**	**F**	**P**
Trial (Tr)	7	1594.82	2.91	0.007	817.16	2.90	0.008
Pre-exposure to alarm cue (PrA)	1	14437.83	26.37	<0.001	12500.61	44.44	<0.001
Pre-exposure to tench cue (PrT)	1	8885.48	16.23	<0.001	11009.90	39.14	<0.001
Tench cue during behavioural assay (Te)	1	25262.17	46.15	<0.001	13111.73	46.61	<0.001
Tr x PrA	7	1048.81	1.92	0.072	774.09	2.75	0.011
Tr x PrT	7	441.83	0.81	0.583	263.74	0.94	0.480
Tr x Te	7	447.94	0.82	0.574	298.71	1.06	0.392
PrA x PrT	1	5206.34	9.51	0.003	7548.35	26.83	<0.001
PrA x Te	1	3148.68	5.75	0.018	4556.87	16.20	<0.001
PrT x Te	1	2242.74	4.10	0.045	4800.66	17.06	<0.001
Tr x PrA x PrT	7	245.29	0.45	0.870	143.28	0.51	0.826
Tr x PrA x Te	7	504.09	0.92	0.493	327.93	1.17	0.327
Tr x PrT x Te	7	306.20	0.56	0.788	218.58	0.78	0.608
PrA x PrT x Te	1	1487.72	2.72	0.102	2985.55	10.61	0.001
Tr x PrA x PrT x Te	7	237.55	0.43	0.879	170.45	0.61	0.750
Total	191						

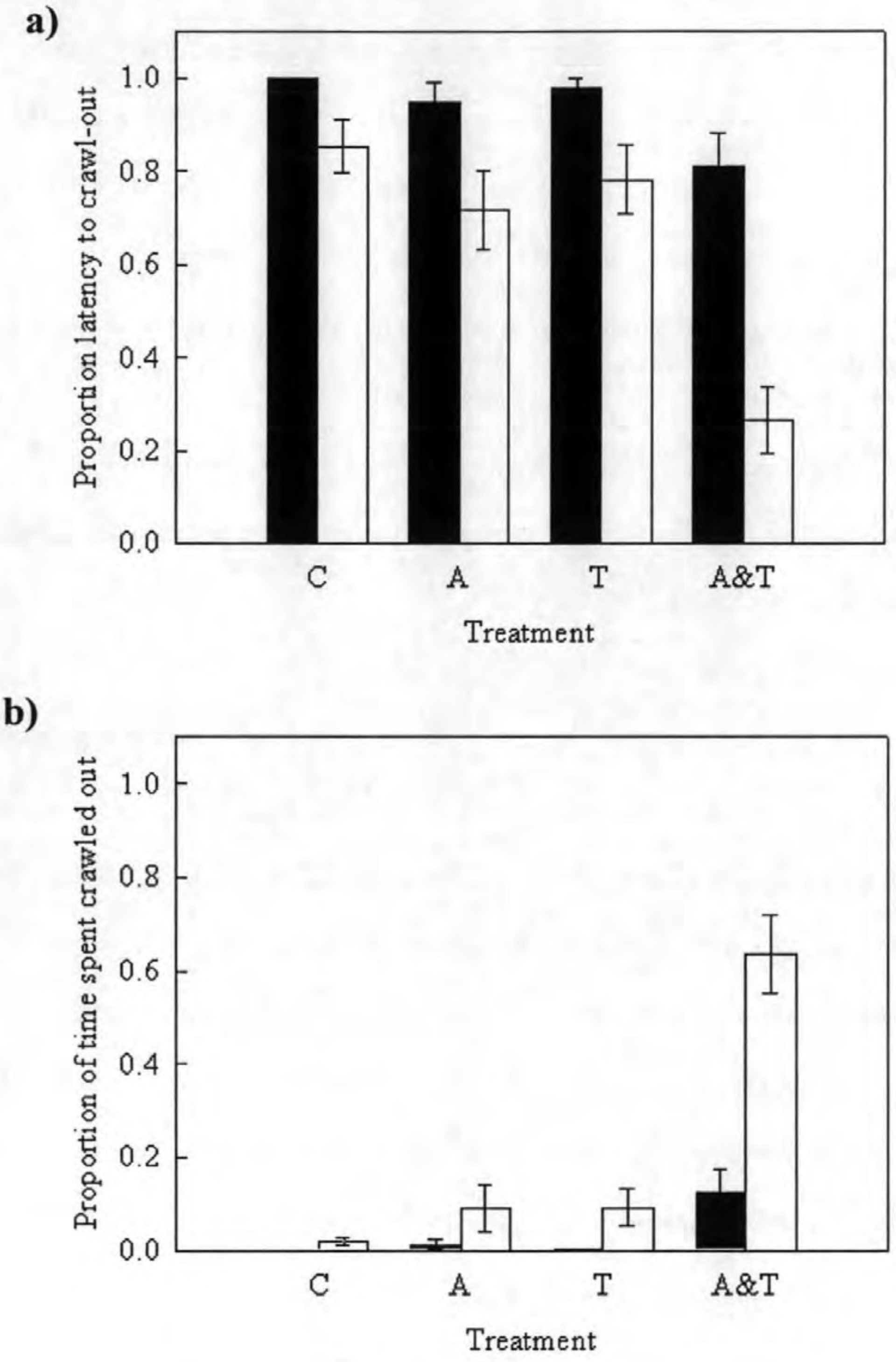

Figure 2.2: The mean (± S.E.) proportion; (a) latency to crawl-out and (b) time spent out of the water by *L. stagnalis* responding to control (dark bars) and predator cue (light bars) during behavioural assays (duration 2 hours), following prior exposure to: control (C), alarm cues alone (A), predator odour alone (T) and alarm plus predator cues (A&T).

Persistence of Cue Association

Latency was always decreased when predator cues were present during the behavioural assay compared to control, irrespective of the conditioning treatment (Fig. 2.3a; Table 2.3; SNK test: $P < 0.05$). However, pre-exposure to predator plus alarm cues significantly decreased the latency to crawl-out in response to predator cue during behavioural assays up to and including the eight days duration compared to individuals pre-exposed to control (Fig. 2.3a; Table 2.3; SNK test: $P < 0.05$). Pre-exposure treatment had no significant effect when snails were exposed to control during the behavioural assay, and duration since pre-exposure to cues did not significantly affect the latency to crawl-out (Table 2.3).

The proportion of time spent crawled out was significantly greater in response to predator cue when *L. stagnalis* had been previously exposed to predator plus alarm cues compared to any of the other treatments (Fig. 2.3b; Table 2.3; SNK test: $P < 0.05$). This significant increase in crawl-out response to predator cues persisted for 8 days following exposure to alarm and predator cues compared with the other treatments. However, the magnitude of the response seen decreased with duration from pre-exposure, with the proportion of time spent crawled out being significantly lower 8 days following pre-exposure compared to only 24 hours following pre-exposure (Table 2.3; SNK test: $P < 0.05$). The number of individuals showing crawl-out behaviour in both control and predator groups showed no significant decline between 24 hours and 8 days (control followed by control; G-test: $G_2 = 5.565$, $P = 0.062$: control followed by predator cue; G-test: $G_2 = 0.208$, $P = 0.901$: alarm plus predator followed by control; G-test: $G_2 = 1.013$, $P = 0.602$: alarm plus predator followed by predator; G-test: $G_2 = 2.672$, $P = 0.263$).

Table 2.3: Latency to crawl-out and proportion of time spent crawled out in response to exposure to tench cues following pre-exposure to tench plus alarm cues at varying durations, 24 hours, 4 days and 8 days prior to behavioural assay. Trial is excluded from model as no significant effect was found using a General Linear Model. N = 23 for all treatment combinations

		Latency			Proportion of time		
Source	**DF**	**MS**	**F**	**P**	**MS**	**F**	**P**
Duration (Du)	2	1691.19	2.97	0.053	1403.60	4.07	0.018
Pre-exposure to alarm plus tench cue (Pr)	1	12189.19	21.40	<0.001	7067.22	20.51	<0.001
Tench cue during behavioural assay (Te)	1	57709.32	101.33	<0.001	25736.90	74.71	<0.001
Du x Pr	2	247.89	0.44	0.648	950.91	2.76	0.065
Du x Te	2	653.22	1.15	0.319	1353.53	3.93	0.021
Pr x Te	1	8583.39	15.07	<0.001	6589.99	19.13	<0.001
Du x Pr x Te	2	225.54	0.40	0.674	1452.64	4.22	0.016
Total	275						

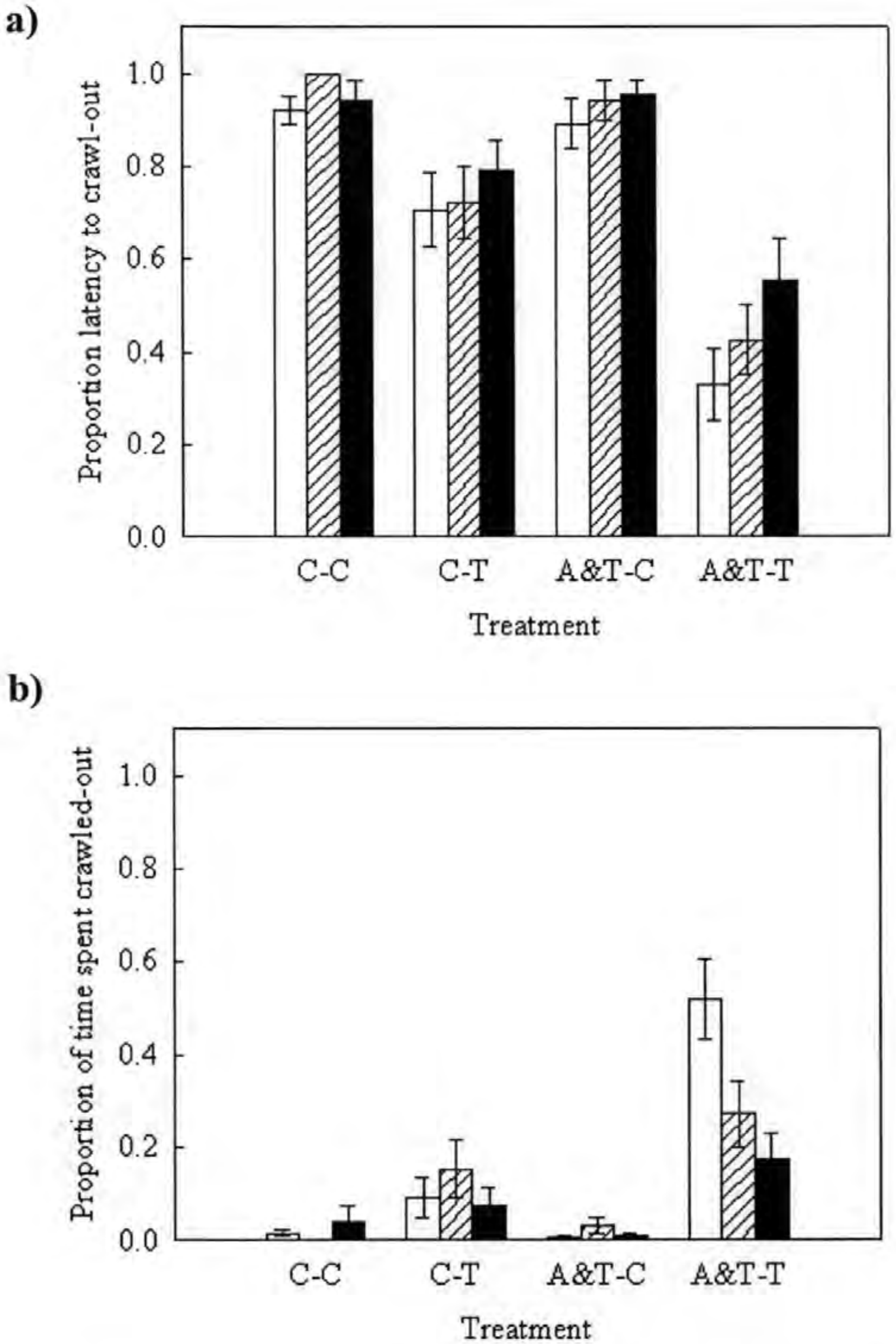

Figure 2.3: The mean (± S.E.) proportion; (a) latency to crawl-out and (b) time spent crawled out by *L. stagnalis* during behavioural assays (duration 2 hours), 24 hours (light bars), 4 days (hatched bars) and 8 days (dark bars) following prior exposure to control (C) or alarm plus predator cues (A&T) in response to either control (C) or predator cue (T) during behavioural assays.

Discussion

The anti-predator response of naïve laboratory reared F1 *L. stagnalis* to predator cue alone demonstrates that this snail species has an innate ability to recognise *T. tinca* kairomones and respond using anti-predator behaviour. Innate responses to predator threat can be due to genetic adaptation to recognise potential predators (Åbjörnsson et al. 2004) or transgenerational influence on offspring (Agrawal et al. 1999). The duration that adults were held in the laboratory prior to production of F1 juveniles for experimentation potentially reduced the effect of maternal experience on the offspring behaviour. However, transgenerational effects of maternal exposure to a dipteran phantom midge predator, *Chaoborus flavicans*, on offspring morphology have been found to persist in the F2 generation of *Daphnia cucullata* (Agrawal et al. 1999).

There was no anti-predator response to alarm signals from crushed conspecifics alone (Fig. 2.1), as found in previous studies on gastropod molluscs (McCarthy and Dickey 2002, Ichinose et al. 2003). Alarm cues may not provide enough information about the type of risk to *L. stagnalis* to induce a response, alternatively they may not be perceived as being associated with a risk of predation at all. However, there was an increase in the proportion of individuals crawling out, as well as time spent out of the water when alarm cues were presented in combination with predator cues compared with predator cue alone. This shows that alarm cues can be identified by *L. stagnalis*, and probably increases the perceived risk in combination with predator cues compared to predator cues alone. An increase in anti-predator response with perceived risk has been found in a number of aquatic species in relation to the predator threat, such as predator density (Ramcharan et al. 1992, Wiackowski and Staronska 1999), distance from prey (Turner and Montgomery 2003), number of prey consumed in the immediate environment (Van Buskirk and Arioli 2002), size of the predator (Kusch et al. 2004) and also with prey vulnerability (Alexander and Covich 1991, Ramcharan et al. 1992, DeWitt et al. 1999, Rundle and Brönmark 2001, Cotton et al. 2004). These factors may combine to increase the accuracy of the anti-predator response in relation to the risk to the prey.

Previous experience of crushed conspecific (alarm cues) plus predator odour from *T. tinca* induced an increase in anti-predator behaviour on subsequent presentations of *T. tinca* cue alone, equivalent to that seen in response to tench plus alarm cues combined during the first experiment (Figs. 2.1 & 2.2). No significant increase in anti-predator behaviour was seen in response to pre-exposure to alarm cues or predator cues alone prior to behavioural assays. This suggests that *L. stagnalis* forms a cue association between predator cue and an alarm substance, which then increases the perceived risk associated with *T. tinca* kairomones on subsequent encounter. Cue association between predator kairomones and damaged conspecifics may provide a more accurate assessment of potential risk from a predator, as it gives information about the current diet of that predator. For instance, the whelk, *Buccinum undatum* increased its escape response over a period of four days exposure to a starfish, *Leptasterias polaris*, feeding on whelks compared to exposure to control or *L. polaris* cues alone (Rochette et al. 1998). The juvenile *B. undatum* used were wild caught, so in this case it is impossible to determine whether there was an innate response to the predator cue. It is likely that they had previously encountered *L. polaris* in the field; hence the laboratory experiment was potentially re-enforcing previous experience.

Cue association may be necessary in order that a prey species is able to recognise accurately the threat of predation. The flatworm, *Dugesia dorotocephala*, demonstrates anti-predator behaviour in response to injured conspecifics, but not to a potential predatory fish, *Lepomis macrochirus* (Wisenden and Millard 2001). However following exposure to damaged conspecifics plus *L. macrochirus* odour, *D. dorotocephala* showed anti-predator behaviour in response to *L. macrochirus* odour alone two days later. As *L. stagnalis* responded to predator cues to a lesser extent without cue association being necessary, it seems likely that this population relies on cue association to give current information on predator risk rather than to identify the predator *per se*.

Cue association has also been shown to be an integral part of predator defence in most fish species studied (Utne and Bacchi 1997, Mirza and Chivers 2001b, Brown 2003, Kelley and Magurran 2003, Larson and McCormick 2005), though Arctic charr, *Salvelinus alpinus*, have been shown to demonstrate innate as well as learnt recognition of potential predators (Vilhunen and Hirvonen 2003). Naïve *Physella virgata* showed induced morphological changes in response to cues from six different sunfish species, which were thought to be in part due to changes in behaviour (Langerhans and DeWitt 2002). However, they were unable to respond differentially to predators depending on their potential risk as they were unable to differentiate between molluscivorous and non-molluscivorous species.

Other studies that have found a response to predator cues alone usually concern wild caught animals, where the history of chemical exposure is unknown, or the animals respond to predator known to be present in their environment (Hopper 2001, Dahl and Peckarsky 2002, Åbjörnsson et al. 2004, Keppel and Scrosati 2004). As many species are capable of learning about potential predators, observed responses may be due to past experience.

Cue association appeared strongest at 24 hours post-exposure, and showed a slight decline over time from 24 hours to 8 days post-exposure. At 8 days post-exposure the proportion of time spent crawled out was still significantly greater in the group previously exposed to predator plus alarm cues, suggesting that memory of the learned cue association persists for at least 8 days following exposure. Although the encounter rate between *T. tinca* and *L. stagnalis* in the source population is unknown, it seems feasible that 8 days is an ecologically relevant time scale in the field. The response to pumpkinseed fish, *Lepomis gibbosus* by *Physa acuta* declined with both distance from the predator and cue age (Turner and Montgomery 2003). However, it is not known whether the decline with cue age, showing a mean behavioural lifetime of 41 hours, was due to an increase in perceived time since a predation event or due to a decline in cue concentration *per se*. The decline over time in anti-predator response by *L. stagnalis* implies a reduction in the perceived risk from *T. tinca* with time since experience of predation cues. This is potentially due to the simulated rate of predation on conspecifics affecting the risk perceived by *L. stagnalis* (Van Buskirk 2002).

The ability to learn about potential predators may be important in responding to invasive predators, or to a new predator regime if the prey species is expanding their range or simply when a predator has not been previously encountered. The crayfish, *Orconectus virilis*, *Procambrus clarkii*, *O. rusticus* and *Austropatmobius pallipes* were all found to be able to form cue association between a pseudopredator, the common goldfish, *Carassius auratus*, and damaged conspecifics (Hazlett et al. 2002). The duration of this cue association varied between species, but in *P. clarkii* was still present after 3 weeks. Individuals that had not been exposed to *C. auratus* cue in combination with damaged conspecific showed no anti-predator response to *C. auratus* cue on subsequent exposure.

A response caused by a random pairing of unrelated cues inducing anti-predator behaviour could prove very costly to prey species. Potentially this is why an anti-predator response declines over time, when no re-enforcement of the cue association occurs. There is also the potential to prevent cue association through learned irrelevance, where cues are presented in a random order so that the organism learns that they are not associated prior to concurrent presentation (Baker et al. 2003, Hazlett 2003), though so far there is no evidence that this would occur in response to a natural predator.

Lymnaea stagnalis has been used previously to demonstrate the neuronal basis of cue association learning (Ito et al. 1999, Benjamin et al. 2000), although these authors used highly artificial stimuli rather than predator cues occurring in the natural environment. *L. stagnalis* has also been used previously to examine the effects of predator cues on anti-predator behaviour and phenotypic plasticity (Rundle and Brönmark 2001, Rundle et al. 2004), however this is the first study to demonstrate the ability of a freshwater gastropod to learn about predation risk. The results of this study concur with those of Rochette et al. (1998) who examined the response of a marine gastropod to cue association, although their study did not consider the effect of prior exposure to alarm cues alone or the prior experience of their wild-caught snails. The present data therefore indicate that the mechanism of cue association learning may be similar in these diverse gastropod taxa.

Cue association learning, combined with variation in response to different types of cue, as demonstrated in this study, could allow individuals to assess real and immediate risks from potential predator species in their environment. The observed decline in response with lack of re-enforcement would also allow organisms to exhibit avoidance behaviours only in the face of active predators and high predation risk, and to reduce costs associated with unnecessary escape behaviour. Such fine tuning of behavioural responses to environmental cues may be highly adaptive, and the precise mechanisms involved represent an intriguing area for future research.

CHAPTER 3

Predator regime influences innate anti-predator behaviour in the freshwater gastropod *Lymnaea stagnalis*.

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Abstract

Predation incurs high fitness costs in aquatic organisms either through direct consumption or inappropriate avoidance responses that reduce time for activities such as feeding and reproduction. Hence, avoidance responses of aquatic organisms should vary to closely match the predation threat in their environment. The freshwater gastropod *Lymnaea stagnalis* occurs in a variety of environments which vary in the presence or absence of predatory fish. I used naïve snails reared from six populations of this species experiencing different predator regimes (three co-occurring with molluscivorous fish and three without) to assess whether populations differed in the type and degree of their avoidance behaviours. Innate behavioural responses to four treatments (control, conspecific alarm cues, fish (*Tinca tinca*) kairomones, and fish kairomones paired with alarm cue) were compared in laboratory trials. The primary anti-predator behaviour of *L. stagnalis* in response to fish kairomones was to crawl out of the water rather than seek refuge under water. This response was strongest when fish kairomones were paired with alarm cues, and varied depending on population origin; snails reared from populations co-occurring with predatory fish showed a stronger response than those raised from populations not experiencing such predators. In addition, populations co-occurring with predatory fish responded to the fish kairomones presented alone. Our findings suggest that the degree of innate anti-predator behaviour shown by *L. stagnalis*, both in terms of the level of risk to which it responds and the degree of response, varies depending on the predator regime experienced by field populations. Together with previous work on cue association, this demonstrates that this species of gastropod is able to match its avoidance behaviour very closely to short and long term predation threats within its habitat.

Introduction

Predators can impose high fitness costs on their prey species, both directly through predation events and also indirectly, either by modifying prey behaviour reducing opportunities to feed or reproduce (Lima and Dill 1990) or by imposing costs through plastic morphological responses (DeWitt 1998, Caramujo and Boavida 2000, Van Buskirk 2000). Changes in morphology and behaviour in response to one predator may also make prey organisms more vulnerable to alternative predator species (Sih et al. 1998). In freshwater systems a number of studies have demonstrated strong responses to predation cues (Reviewed in: Dodson et al. 1994, Chivers and Smith 1998, Lass and Spaak 2003, Werner and Peacor 2003), however many aquatic prey species occupy a wide variety of habitats and experience varying levels of predation threat, such that a single mode of behavioural response is unlikely to be an effective method of avoiding all potential predators. As such, I would predict that anti-predator behaviour would vary between populations with different predator regimes to closely match the type and level of predation threat present (McPeek 1990).

Local adaptation to predation threat is likely to occur in relatively sedentary species with low dispersal abilities, whereas induced responses are more likely in species which have potential for high dispersal between generations, as parents and offspring are more likely to encounter alternate predator regimes (Kawecki and Ebert 2004). Adaptation may also occur where plastic responses are costly if the lag between expressing one phenotype or its alternative is too far behind the changes in the environment (Padilla and Adolph 1996), information gathering to produce the correct phenotype is costly (DeWitt 1998) or costly responses to non-threatening species are likely (Langerhans and DeWitt 2002).

In freshwater organisms induced responses to predation cues have been found where species have terrestrial stages allowing dispersal between alternate habitats. For example mayfly larvae from fishless streams exhibit behavioural responses *(Baetis rhodani* (Pictet, 1844): Tikkanen et al. 1996) and alter their life history traits (*Baetis bicaudatus* Dodds, 1923: Peckarsky et al. 2002, *Ephemerella invaria* (Walker, 1853): Dahl and Peckarsky 2003) in response to fish cues. However, in tadpoles of wood frogs, *Rana sylvatica* LeConte, 1825 (Relyea 2002b), red-legged frogs, *Rana aurora* Baird and Girard, 1852 (Kiesecker and Blaustein 1997), and moor frogs, *Rana arvalis* Nilsson, 1842 (Laurila et al. 2006) population adaptation to predator regimes occurred despite the potential of adults to disperse between habitats. Laurila (2000) found no innate differences between populations in tadpoles responses to predator cues of the common frog, *Rana temporaria* Linnaeus, 1758. He suggested that the reliance on plastic responses in this species may be due to a lack of genetic variation on which selection could act, variability in the predation regime that populations are exposed to or possibly due to adult dispersal between populations preventing local adaptation.

Local adaptation to predators has also been shown in freshwater species with low dispersal abilities, where the offspring experience the same environment as the parent. For example the Trinidadian guppy, *Poecilia reticulata* Peters, 1859, shows adaptive responses to food levels (Bashey 2006) and predator regime (O'Steen et al. 2002) and *Gammarus pulex* (Linnaeus, 1758) shows innate behavioural variation between populations exposed to differing predator regimes (Åbjörnsson et al. 2004). Induced responses have also been found in species with low dispersal, for example some fish species demonstrate an ability to learn about potential predation threat rather than demonstrating local adaptation and innate recognition (Reviewed in: Brown 2003), possibly because the costs of information gathering are lower than those of producing inappropriate fixed responses (DeWitt 1998).

Gastropods have been utilised extensively to study the responses to predation threat in aquatic systems. Previous studies do suggest their ability to respond to both conspecific and heterospecific alarm cues (Snyder 1967, Stenzler and Atema 1977) as well as showing differential responses to alternate predation threats both related to the type (Snyder 1967, Turner et al. 1999, Turner et al. 2000, Hoverman et al. 2005) and proximity (Turner and Montgomery 2003) of predators. Experience has also been shown to influence the degree of response to predator cues shown by aquatic gastropods (Chapter 2: Rochette et al. 1998, Dalesman et al. 2006, Turner et al. 2006), and morphological responses have been demonstrated to be plastic (Trussell 2000a, Rundle and Brönmark 2001, Turner and Montgomery 2003, Cotton et al. 2004); the degree of such responses may vary with resource availability such as food (Wojdak and Luttbeg 2005) or calcium concentration (Rundle et al. 2004).

Freshwater gastropods have limited dispersal ability, as they are only able to spend short periods emersed without risking desiccation. As such it may be predicted that populations will be under selection dependent on the predator regime they experience, and this prediction is supported by empirical data (Covich et al. 1994, McCarthy and Fisher 2000, Bernot and Whittinghill 2003). However, these studies have not explicitly tested for differences between populations across replicate sites differing in their predator status. Also, this previous work has used wild-caught animals, which means that any differences between populations may reflect plastic responses rather than local adaptation. Previous studies using laboratory reared animals does suggest that the anti-predator response in freshwater gastropods has an innate element, but can also vary with experience (Chapter 2: Dalesman et al. 2006, Turner et al. 2006).

The great pond snail, *Lymnaea stagnalis*, inhabits a wide range of freshwater habitats, including those with and without predatory fish and might be predicted to exhibit adaptation in response to these differing predator regimes. This species has been demonstrated to have both innate and learnt components in its behavioural response to predator cues (Chapter 2: Dalesman et al. 2006). Here I investigate whether the innate aspects of this behaviour vary between populations experiencing differing predator regimes. F1-generation snails were tested for their responses to cues from a known gastropod predator, tench, *Tinca tinca* (Brönmark 1994), that commonly overlaps in distribution with *L. stagnalis*. Responses to fish kairomones were tested both on their own and concurrently with crushed gastropod cues to simulate a predation event. Hence, I was able to assess how populations differed in their response to the type of cue presented and in the degree of response shown. Adaptation through natural selection has been shown to account for divergence between aquatic populations experiencing differing predation pressure (Cousyn et al. 2001, O'Steen et al. 2002, Meyer et al. 2006). As such I predicted that populations that co-existed with predatory fish would have adapted to show higher innate responses than those that did not experience such predation risk.

Methods

Field collection sites

Adult *L. stagnalis* used to produce experimental snails were collected between September 2005 and May 2006 from six sites on the Somerset Levels, an extensive wet meadow system in the southwest UK, covered by a series of interlocking drainage ditches and channels (Figure 3.1). The three sites with predatory fish present (South Drain - 51.18N 2.88W, Sowy River - 51.07N 2.88W, North Moor - 51.07N 2.96W) were large channels (width: 6-22m, depth: 0.7-1.1m). Information from the local angling club, Taunton Angling Association (pers. com.), was used to confirm the presence of tench at these sites. The three other sites (Chilton Moor - 51.19N 2.88W, Little Hook - 51.06N 2.87W, Wistaria Farm - 51.07N 2.98W) were small ditches (width: 1.8-2.9m, depth: 0.45-0.7m); electro-fishing was used to confirm the absence of molluscivorous fish. Large and small sites were located in a pair-wise fashion such that each small site was closer to a large site than to the nearest small site and vice versa to reduce any potential influence of geographic location. Repeated sampling at all the sites between March 2004 and September 2006 indicated that *L. stagnalis* is common at these sites for most of the year.

Study Organisms

In the laboratory, adult *L. stagnalis* were maintained in aquaria with 4 l of aerated artificial pond water (ASTM 1980) with 90 mgl^{-1} [Ca^{2+}] (Rundle et al. 2004) at 15±1 °C under a 12:12 light:dark cycle, and fed on Iceberg lettuce and spinach. To minimize the influence of adult experience on juvenile development and behaviour, the F1 snails (6±0.5mm spire height) used as responders were bred from egg masses laid a minimum of two months post collection. They were then maintained separately in the same conditions as the adults. Tench used to produce predator cue were kept in analogous condition to the snails, and fed Nutri-flake (Hozelock Cyprio, U.K.), which contains no mollusc extracts, and hence avoided potential dietary cues affecting the trials.

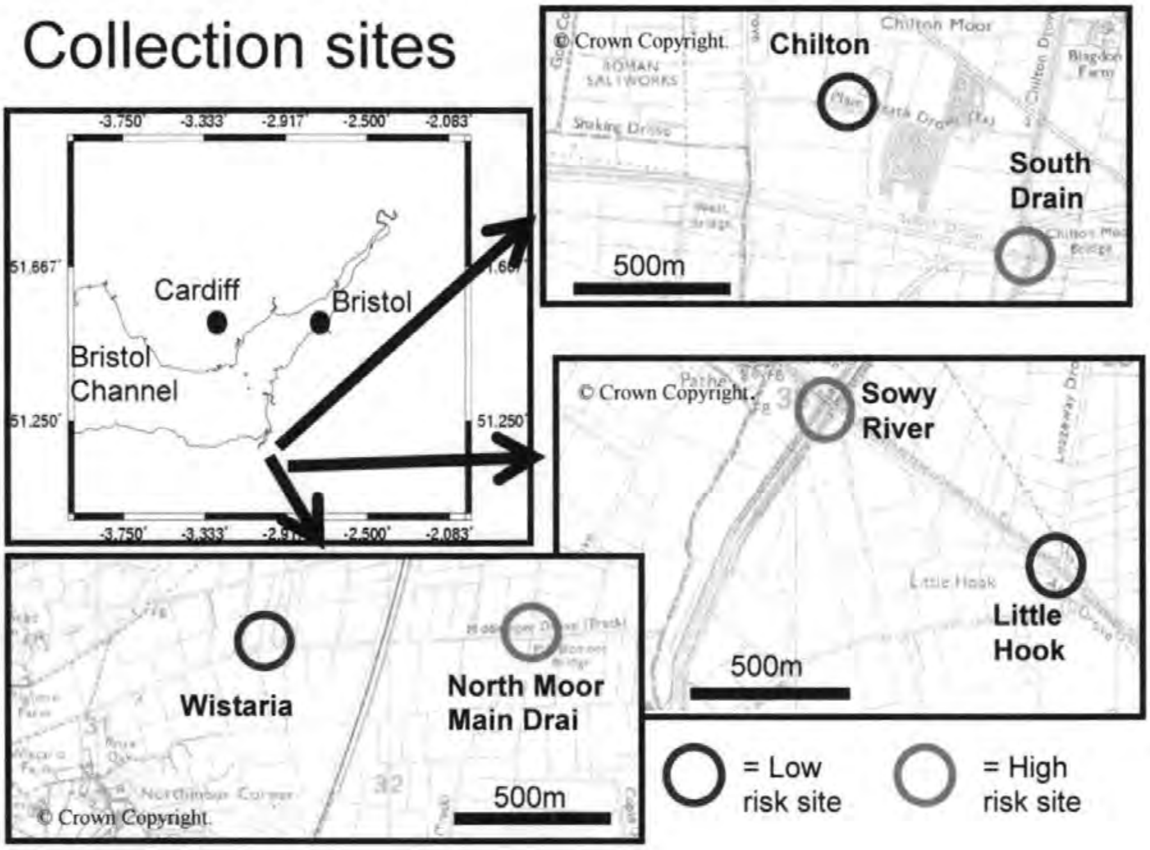

Figure 3.1: Locations of collection sites on the Somerset Levels.

Odour production

Predator kairomones and alarm cues were produced in the same way in all the experiments (Chapter 2: Dalesman et al. 2006). Four exposure treatments were used: (i) fish kairomone water, produced by placing three tench (length 10±1 cm) into 4 l of aerated artificial pond water for 1 hour; (ii) alarm cue, produced by mixing three crushed snails (6±0.5 mm) into 4l of aerated artificial pond water; (iii) fish kairomone plus alarm cue, produced by mixing three crushed snails (6±0.5 mm) into 4 l of tench cue water; and (iv) the control which was aerated artificial pond water.

Behavioural Assay

Behavioural trials were carried out in the same way for all experiments under laboratory conditions analogous to those in which the snails were maintained (Chapter 2: Dalesman et al. 2006). 24 individuals were used for each treatment combination, giving a total of 96 individuals per population, 576 in total. Behavioural chambers consisted of a white plastic dish, 165mm diameter x 60mm depth (A.W.Gregory & Co. Ltd., U.K.), with a longitudinally sectioned white PVC pipe (36mm long, 30mm diameter) attached open side down to the centre using non-toxic sealant (Wickes Ultimate Sealant and Adhesive, Wickes Building Supplies Ltd., U.K.) to provide a refuge. Juvenile snails were acclimated to behavioural chambers for 24 hours in 630ml of artificial pond water prior to behavioural assays. 70ml of cue water was added to each chamber at the start of the behavioural assay to give a final concentration of 10% cue water. The position of each snail within the behavioural chambers was recorded every 5 minutes for 2 hours following cue addition. Crawl-out behaviour has been found to be the main anti-predator response of *L. stagnalis* to tench cue by snails originating from a population with fish predators present (Chapter 2: Dalesman et al. 2006), however use of a refuge by *L. stagnalis* may occur in response to fish predators (Rundle and Brönmark 2001), so both types of behaviour were analysed here using two variables: the proportion of total time spent in avoidance behaviour; and the latency to avoidance, calculated as the proportion of total time during the trial before an avoidance behaviour was observed (Cotton et al. 2004).

Data Analysis

Data were analysed using ANOVA in GMAV5 (Institute of Marine Ecology, University of Sydney, Sydney, Australia). Alarm cue, tench cue, habitat type (predatory fish present or absent) and population (nested within habitat type) were used as factors in the analysis. Both time spent in avoidance and latency to avoidance were calculated as proportions of total duration, and were arcsine-square root transformed prior to analysis (Sokal and Rohlf 1995). Student-Newman-Keuls (SNK) tests were used to carry out post-hoc pair-wise comparisons.

Results

The treatment with a combination of tench kairomones plus alarm cue produced the greatest avoidance response, with the longest time spent crawled out for all populations. This response was significantly higher for individuals bred from adults that originated from populations experiencing fish predation (Fig. 3.2a, Table 3.1: $F_{1,4} = 16.66$, $P = 0.015$, SNK: $P<0.01$). In addition, snails originating from populations experiencing fish predation also showed a significant response to the fish cue treatment, though this was significantly lower than the response to combined cues (SNK: $P<0.05$); individuals from populations where no fish predators were present showed no significant crawl-out response to fish cues alone. None of the populations showed a significant crawl-out response to alarm cues in isolation.

The latency to crawl-out was significantly shorter in response to tench kairomones and alarm cues combined compared with either cue presented alone for all the populations tested, and did not differ depending on habitat origin (Fig 3.2b: table 3.1: $F_{1,4} = 194.13$, $P<0.001$). However, the response to tench kairomones presented alone did differ significantly between habitats, with latency being significantly lower in populations originating from areas with fish predators present (Fig. 3.2b: table 3.1: $F_{1,4} = 76.08$, $P = 0.001$: SNK: $P<0.01$).

The time spent under the refuge significantly decreased in response to the presence of tench cue (ANOVA: main effect of tench cue: $F_{1,4} = 13.90$, $P = 0.020$) but was not affected by any other factors.

Table 3.1: Results from 4-way nested ANOVA on time spent crawled out of the water and latency to crawl out. Factors used are: habitat type (predatory fish/no predatory fish), population (nested in habitat type), alarm cue (present/absent) and tench cue (present/absent). N = 24 for all treatment combinations.

Source	**d.f.**	**Proportion of time**			**Latency**		
		Mean square	**F**	**P**	**Mean square**	**F**	**P**
Habitat type (Ha)	1	3.41	27.76	0.006	4.10	30.61	0.005
Population nested in habitat (Po)	4	0.12	1.94	0.103	0.13	1.27	0.282
Alarm cue (A)	1	14.61	266.87	<0.001	13.91	248.47	<0.001
Tench cue (T)	1	24.62	222.23	<0.001	31.16	416.26	<0.001
Ha*A	1	0.73	13.27	0.022	0.35	6.25	0.067
Ha*T	1	3.72	33.57	0.004	5.70	76.08	0.001
A*Po	4	0.05	0.86	0.486	0.06	0.53	0.714
T*Po	4	0.11	1.75	0.138	0.07	0.71	0.586
A*T	1	14.24	355.51	<0.001	14.05	194.13	<0.001
Ha*A*T	1	0.67	16.66	0.015	0.13	1.80	0.251
A*T*Po	4	0.04	0.63	0.640	0.07	0.69	0.602
Total	575						

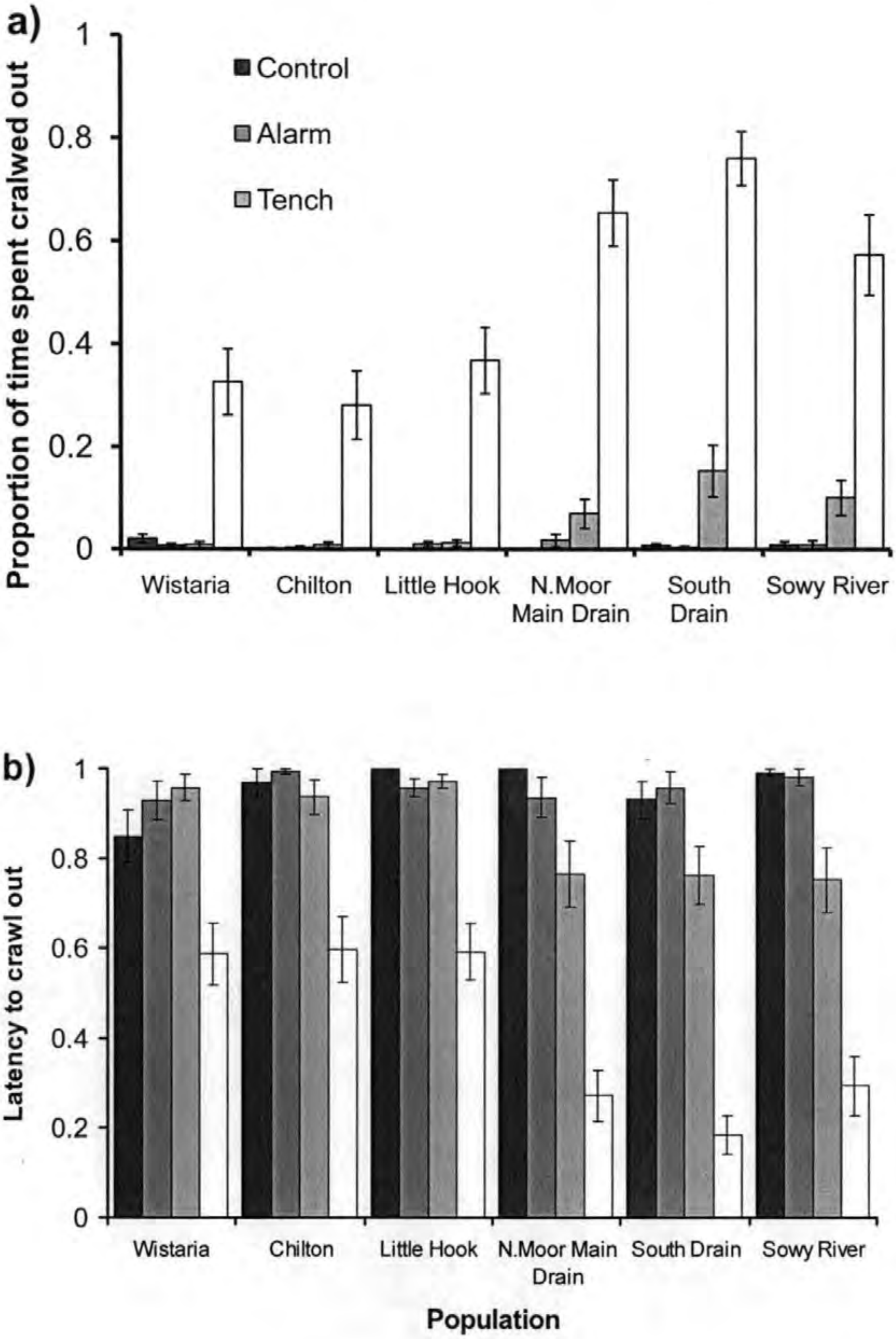

Figure 3.2: The mean (± s.e.) crawl out response of F1 *L. stagnalis* from populations with low (Wistaria; Chilton; Little Hook) and high (N. Moor Main Drain; South Drain; Sowy River) predatory fish regimes responding to different treatment levels, showing a) proportion of time spent crawled out and b) latency to crawl out. N=24 for each treatment combination.

Discussion

All the populations used in this study showed a degree of innate anti-predator response to predator kairomones presented in combination with alarm cues by crawling above the water line. This indicates that the populations not experiencing fish predation are still able to recognise the predation threat from tench and respond with appropriate behaviour. The response seen from fishless populations, however, is significantly lower that seen from populations exposed to predatory fish, however, suggesting that the innate perceived threat is lower in the fishless populations. The populations from habitats with predatory fish present also demonstrated an innate response to tench cues alone, not seen in the populations without any predatory fish in their natural habitat. The decrease seen in use of the refuge in response to tench cues is most likely to be an artefact of the increase in crawl out response to this cue, confirming that crawl out behaviour is the main response to tench kairomones shown by *L. stagnalis* as found in a previous study (Chapter 2: Dalesman et al. 2006).

The difference found between high and low risk populations in a laboratory reared F1 generation suggest that adaptation to local predator regimes has occurred in the six populations studied. This result concurs with results on responses from other freshwater organisms with limited dispersal such as *Gammarus pulex* (Åbjörnsson et al. 2004) and the Trinidadian guppy, *Poecilia reticula* (O'Steen et al. 2002), which also showed divergence in the degree of innate response to predatory fish cues relative to the predators present in their environment. As all three of these species have relatively low dispersal abilities these results support the theory that species with low dispersal capabilities adapt to local predator regimes rather than relying purely on plasticity to alter phenotype (Kawecki and Ebert 2004).

The reduced response to fish cues from populations not normally exposed to predatory fish suggest that *L. stagnalis* does not have a general anti-predator response to all potential predator species it could encounter, as these populations showing low responses to predatory fish cues are exposed to large numbers of invertebrate predators (Dalesman, pers. obs.) whose numbers would be reduced by predatory fish elsewhere (Åbjörnsson et al. 2002). Indeed, it has been previously demonstrated that aquatic gastropods are able to vary their response depending on predator identity (Snyder 1967, Turner et al. 2000), so potentially populations may lose their response to one type of predator without reducing their response to another (Mikolajewski et al. 2006). This may eventually result in the complete loss of the ability to respond to a potential predator by a prey species if they no-longer come into contact (Stoks et al. 2003). The reduced avoidance response in low risk populations suggests that there may be costs associated with retaining anti-predator behaviour towards fish. Traits linked with an increased tendency to respond to fish cues may incur costs when expressed in fishless environments, for example the loss of sexual ornamentation in fish (Magurran 1999, Basolo and Wagner 2004) or the costs associated with maintaining systems to show plastic responses (DeWitt 1998).

Assuming that costs are associated with maintaining the high degree of anti-predator behaviour in response to fish cues when predatory fish are not present, it seems perplexing that the response is retained at all in the low risk populations studied here. One explanation may be that fishless environments can be invaded by predatory fish during times of flooding, as the fish and fishless populations used here are in relatively close proximity (<1km), and on a flood plain, there is a possibility that this may occur. The high and low response populations are connected by a series of drainage ditches so there is also potential for gene flow to occur between populations preventing a complete loss of anti-predator behaviour from the fishless populations. As the fitness consequences of not responding to fish correctly are likely to be much higher than occasional inappropriate behaviour when fish are not present, then it is possible that even infrequent encounters with predatory fish may result in the behaviour being retained. An alternative explanation would be that at least part of the response seen to predatory fish cues is due to a general sensitivity to predators rather than a predator specific response, or linked within a suite of behavioural syndromes (Sih et al. 2004). In this case the 'low risk' populations are still exposed to an assortment of invertebrate predators including beetles, bugs and leeches (Dalesman, pers. obs.), and elements of the response seen to fish kairomones may be included in the anti-predator response to these other taxa.

The nature of the environment in which the snail populations used in this study live, with the close proximity and potential for migration along linked water systems, suggests that the differences seen will be under strong selection, both for high response under threat of predation, but also to lose responses when that threat is no longer present. The degree of variation seen in both population groups, with a few individuals from the populations lacking predatory fish showing a high response to fish and alarm cues, and vice versa, indicates potential within the population to react rapidly to a change in predatory threat (Fisher 1930). Rapid microevolution causing divergence between conspecific populations (Hendry and Kinnison 1999) has been demonstrated elsewhere as a result of natural selection caused by predators (Cousyn et al. 2001, O'Steen et al. 2002, Losos et al. 2004, Meyer et al. 2006, Nosil and Crespi 2006).

In summary this study suggests that innate differences in behaviour and morphology found in other aquatic species (O'Steen et al. 2002, Åbjörnsson et al. 2004) may be generalised across freshwater taxa with poor dispersal abilities. The inability to move from areas of high predation risk to low predation risk may be promoting adaptive responses to local predator regimes. I have previously demonstrated that *L. stagnalis* is able to alter risk perception of a known predator based on recent experience (Chapter 2: Dalesman et al. 2006). Such abilities to associate predation cues with perceived risk to fine-tune the innate recognition of predation threat in the short term, alongside the long term adaptive response to predators demonstrated in the present study, suggest that freshwater snails such as *L. stagnalis* are able to closely match their anti-predator behaviour to their environment.

Chapter 4

Adaptation of anti-predator behaviour in an aquatic gastropod: insights from artificial selection.

Dalesman, S., Rundle, S. D. & Cotton, P. A. In Review. Adaptation of anti-predator behaviour in an aquatic gastropod: insights from artificial selection. *Oecologia*.

Abstract

Adaptation to predation through changes in life history, physiology, morphology or behaviour often occurs in populations experiencing stable predator regimes. Artificial selection can be used to assess the heritability of these traits and, hence, how they may respond to natural selection. The freshwater gastropod *Lymnaea stagnalis* varies between populations in its innate anti-predator behaviour dependent on the local predator regime by increasing innate crawl out responses to fish cues in populations where predatory fish are present. I used artificial selection to investigate whether such divergence in behaviour between populations is due to local adaptation or transgenerational induction. *Lymnaea stagnalis* responded rapidly to selection for high and low responses to predator cues; divergence in anti-predator behaviour between high and low selection lines over two generations was similar to the range found in natural populations, and heritability for crawl-out behaviour ($h^2 = 0.33$) was comparable to that for behavioural traits found in other taxa. I conclude that differences in innate anti-predator behaviour between high and low risk populations are due to local adaptation, and that such divergence has the potential to evolve within only two generations.

Introduction

Local adaptation will occur when a heritable trait, or a suite of traits, gives an organism a higher fitness relative to alternate genotypes within a particular environment (Brakefield 2003). Selection for particular genotypes may be expressed in one of two ways in the phenotype of an individual, either by controlling the plasticity in response stimuli (Tollrian and Harvell 1999), or potentially by producing a fixed phenotype irrespective of environment (Conner and Agrawal 2005). For example, *Polygonum* spp. show a plastic response by increasing internode distance in response to increased density, and a fixed response in that the node number remains constant (Griffith and Sultan 2006). Genetic adaptation for fixed traits is likely to be favoured where the environment is stable, either temporally or spatially, relative to the distribution of the responding species (Kawecki and Ebert 2004), whereas adaptation favouring plastic traits is more likely in a variable environment where a fixed response is costly to produce or maintain in the absence of necessity (Pettersson and Brönmark 1999, Rigby and Jokela 2000, Langerhans and DeWitt 2002).

Predation incurs high, direct fitness costs on prey organisms, and potentially indirect costs due to inappropriate responses in the absence of a genuine threat (Lima and Dill 1990). Predator presence has also been found to alter prey community dynamics, for example by driving trait divergence between ecotypes in natural populations (*Gasterosteus aculeatus*: Vamosi and Schluter 2004, *Timema* spp.: Nosil and Crespi 2006), and decreasing the impact of competition by reducing prey density (Meyer and Kassen 2007). Hence, there is likely to be strong selection on an organism to respond appropriately to predators in their environment either through local adaptation in fixed innate responses or by exhibiting appropriate induced defences.

Differences between natural conspecific populations are frequently described as being due to alternate predator regimes. There is currently a glut of studies documenting phenotypic plasticity in response to varying predator regimes as a potential cause for these differences (reviewed in: Tollrian and Harvell 1999, Lass and Spaak 2003, Benard 2004, Miner et al. 2005). However, the responses of naïve individuals give an indication that such differences may also be due to local adaptation. For example, innate hiding behaviour of laboratory reared male crickets, *Gryllus integer*, responding to novel cues has a longer duration in a population naturally exposed to higher predation rates (Hedrick and Kortet 2006). The aquatic gastropod, *Lymnaea stagnalis* (Chapter 3: Dalesman et al. 2007b) and *Daphnia pulex* (Åbjörnsson et al. 2004) also show stronger innate responses to fish cues when reared from populations naturally exposed to predatory fish. However, all of these studies used F1 generation laboratory reared animals, so there is also potential that transgenerational induction may account for behavioural differences between populations (Agrawal et al. 1999).

There are some examples of where predation has been found to drive rapid divergent evolution between conspecific populations under natural selection. For example, average limb length in the lizard, *Anolis sagrei*, was found to change in only a few generations following the introduction of a predatory lizard, *Leiocephalus carinatus* (Losos et al. 2006). In *Poecilia reticulata* (Trinidadian guppy) behavioural adaptation to high and low predator environments has been found to occur rapidly under natural conditions in 26-36 generations (O'Steen et al. 2002), brightness of colour spots changes significantly after 10 generations (Endler 1980) and alteration of growth rates in response to low predator regimes occurs within 13 years (Arendt and Reznick 2005).

Similarly, rapid evolution in anti-predator responses can be artificially selected for in laboratory conditions. The duration of death feigning in beetles, *Tribolium castaneum* (Miyatake et al. 2004) and in *Callosobruchus chinensis* (Ohno and Miyatake 2007) responds to artificial selection, and increased duration has been shown to reduce vulnerability to predators. Susceptibility to parasites and parasitoids also responds to artificial selection, for example in the gastropod, *Biomphalaria glabrata* (Webster and Woolhouse 1999) and in the fruit fly, *Drosophila melanogaster* (Kraaijeveld et al. 2001). This type of selection experiment can give an insight into the mechanism by which traits diverge in natural populations, and also give an indication of the potential for adaptation in response to changing environments in natural populations (Brakefield 2003, Fuller et al. 2005).

In aquatic gastropods, evidence has been found for plastic responses to predation cues, in terms of both morphological (DeWitt 1998, Trussell 2000b, Rundle and Brönmark 2001) and behavioural responses (Rochette et al. 1998, Chapter 2: Dalesman et al. 2006, Turner et al. 2006), though there appears to be an innate element to the behaviour as well (Chapter 2: Dalesman et al. 2006, Turner et al. 2006). Innate responses to predation cues by *L. stagnalis* have been found to vary significantly between populations exposed to differing predator regimes (Chapter 3: Dalesman et al. 2007b). However, it is unclear whether this variation was due to local adaptation or transgenerational induction of cues (Agrawal et al. 1999) as the behavioural trials were carried out on the F1 lab reared generation.

Here I use artificial selection to investigate the mechanism by which populations of the pulmonate gastropod, *Lymnaea stagnalis* (L.) differ in anti-predator behaviour. Individuals sourced from two wild populations were used to produce selection lines as there is evidence that conspecific populations may respond differently to uniform selection pressure (Cohan and Hoffmann 1989). If the initial population differences are due to genetic adaptation for a fixed response, then the response to predator cues in the population should persist at a similar level in the laboratory in the absence of selection, but that the rapidity of inducation and duration anti-predator behaviour could be manipulated by selecting for either high or low responding individuals to produce high and low response selection lines respectively.

Methods

Adult great pond snails *Lymnaea stagnalis* (Gastropoda: Pulmonata: Basommatophora) were collected from two adjacent sites on the Somerset Levels, U.K.: South Drain (51.18N 2.88W) and Chilton Moor (51.19N 2.88W) in September 2005. In the laboratory, adults were kept in aquaria with 4 l of aerated artificial pond water (ASTM 1980) with 90 mgl^{-1} [Ca^{2+}] (Rundle et al. 2004). Following acclimation to laboratory conditions at 15°C for three weeks they were transferred into 20±1°C under a 12:12 light:dark cycle, and fed on Iceberg lettuce and spinach. Egg masses used to raise the initial F1 generation were collected a minimum of 2 weeks following transfer into 20±1°C, and maintained in similar conditions to the adults following hatching until juveniles reached 6±0.5mm when they were used for behavioural trials.

Tinca tinca (tench) were used to produce fish kairomones as this species overlaps in distribution and habitat requirements with *L. stagnalis*, and is a known gastropod predator (Brönmark 1994). Fish were maintained in analogous laboratory conditions to *L. stagnalis* and fed Nutri-flake (Hozelock Cyprio, U.K.), which contains no mollusc extracts, and hence avoided potential dietary cues affecting the trials.

Selection of response lines

F1, laboratory-reared snails were used in selection trials to produce the high response and low response lines to breed the F2 generation. Snails were selected based on their responses during a single 2 hour behavioural trial, responding to a combination of tench kairomones and conspecific alarm cue (see 'behavioural trials' below). Individuals were considered 'low response' if they demonstrated no crawl-out behaviour on exposure to combined cues during the 2 hour period, and 'high response' individuals if they crawled out of the water within the first 60 minutes of the trial and did not re-enter the water before the end of the 2 hour trial. From each original population, 24 individuals were chosen based on their response during the behavioural trial for each of the low and high response lines to provide adequate mate choice in producing the F2 generation. Twenty-four individuals were also selected randomly from each of the populations to provide an unselected line against which the selected lines could be compared. Allowing mate choice rather than using restricted pairs may mimic the breeding within a small population more closely and prevents inbreeding depression affecting behavioural responses (Fry 2003, Fuller et al. 2005).

Behavioural trials on F2 generation snails were carried out as for F1 snails, using combined cues, to select individuals to produce the F3 generation. In this case only low response individuals were taken from the low response selection lines, and high response individuals from the high response selection line for each population, with the unselected line continued using randomly chosen offspring from the randomly selected F1 group.

Behavioural trials

Trials were carried out to assess the behavioural response of each selection line in each generation to predatory fish kairomones and alarm cues. The behavioural trials and cue production followed the methodology outlined in detail by Dalesman *et al.* (2006, Chapter 2), with 24 individuals exposed to each of 4 treatments: i) control (artificial pond water alone), ii) alarm cue (crushed conspecific snail), iii) tench kairomone or iv) tench kairomone plus alarm cue. The position of each snail in individual behavioural chambers was recorded for 2 hours following cue addition and crawl-out behaviour was analysed as the main anti-predator response of *L. stagnalis* to fish predation threat (Chapters 2&3: Dalesman et al. 2006, Dalesman et al. 2007b). Both the time spent crawled out during the behavioural trial, and the latency to crawl out calculated as the proportion of total time during the trial before crawl-out behaviour was observed (Cotton et al. 2004), were analysed as the main anti-predator responses of *L. stagnalis* responding to predation cues from tench (Chapters 2&3: Dalesman et al. 2006, Dalesman et al. 2007b).

Data analysis

Behavioural data from the F1 generation were analysed, using a 3-way ANOVA, with population origin (Po: South Drain vs. Chilton), alarm cue (A: presence vs. absence) and tench cue (T: presence vs. absence) used as factors, to assess whether the populations were showing similar responses prior to selection. The F2 and F3 behavioural trials were analysed using a 5-way ANOVA, factors included in the analysis were population origin (Po), generation (Ge: F2 vs. F3), selection line (Se: high vs. unselected vs. low), alarm cue (A) and tench cue (T), to assess the responses to selection. Both time spent in avoidance and latency to avoidance were calculated as proportions of total duration, and were arcsine-square root transformed prior to analysis

(Sokal and Rohlf 1995). All ANOVAs were analysed in GMAV5 (Institute of Marine Ecology, University of Sydney, Sydney, Australia), using Student-Newman-Keuls (SNK) tests to carry out post-hoc analyses.

In order to assess whether the effects of inbreeding or genetic drift had occurred following selection the variance in behavioural responses was analysed using Levene's test for equal variances for both latency to crawl out and time spent crawled out in each population. The conservation of variance between generations would imply that there is still variation on which selection can act (Fisher 1930).

Heritability (h^2) of the crawl out response was calculated using the formula:

$$h^2 = R/S$$

where R is the response to selection (the per generation change in the mean of the trait) and S is the selection differential (difference between the mean for the entire measured population and the mean response of those selected; (Conner 2003). Heritability was calculated as the mean response over two generations for each population.

Results

Behaviour of the F1 generation

For F1 generation snails the time spent crawled out and latency to crawl out did not differ between populations (see Fig 4.1.), however there was a significant effect of treatment; the time spent crawled out was significantly greater and latency to crawl out was significantly shorter in response to both cues presented together compared to control or either cue presented alone for both populations (Fig. 4.1: Table 4.1: A*T: for

a) time spent crawled out: $F_{1,184} = 60.44$, $P < 0.001$; b) latency to crawl out: $F_{1,184} = 43.01$, $P < 0.001$).

Table 4.1: Results from 3-way ANOVA on F1 generation snails, showing time spent crawled out and latency to crawl out. Factors included are: population (Chilton/South Drain), alarm cue (present/absent) and tench cue (present/absent). N = 24 for all treatment combinations.

		Proportion of time			**Latency**		
Source	**d.f.**	**Mean square**	**F**	**P**	**Mean square**	**F**	**P**
Population Po)	1	0.015	0.17	0.677	0.068	0.62	0.431
Alarm cue A)	1	5.587	66.62	<0.001	5.354	48.95	<0.001
Tench cue T)	1	7.474	89.13	<0.001	8.289	75.77	<0.001
Po*A	1	0.007	0.09	0.768	0.001	0.01	0.941
Po*T	1	<0.001	<0.01	0.969	<0.001	<0.01	0.991
A*T	1	5.068	60.44	<0.001	4.705	43.01	<0.001
Po*A*T	1	0.039	0.47	0.495	0.085	0.78	0.379
Total	191						

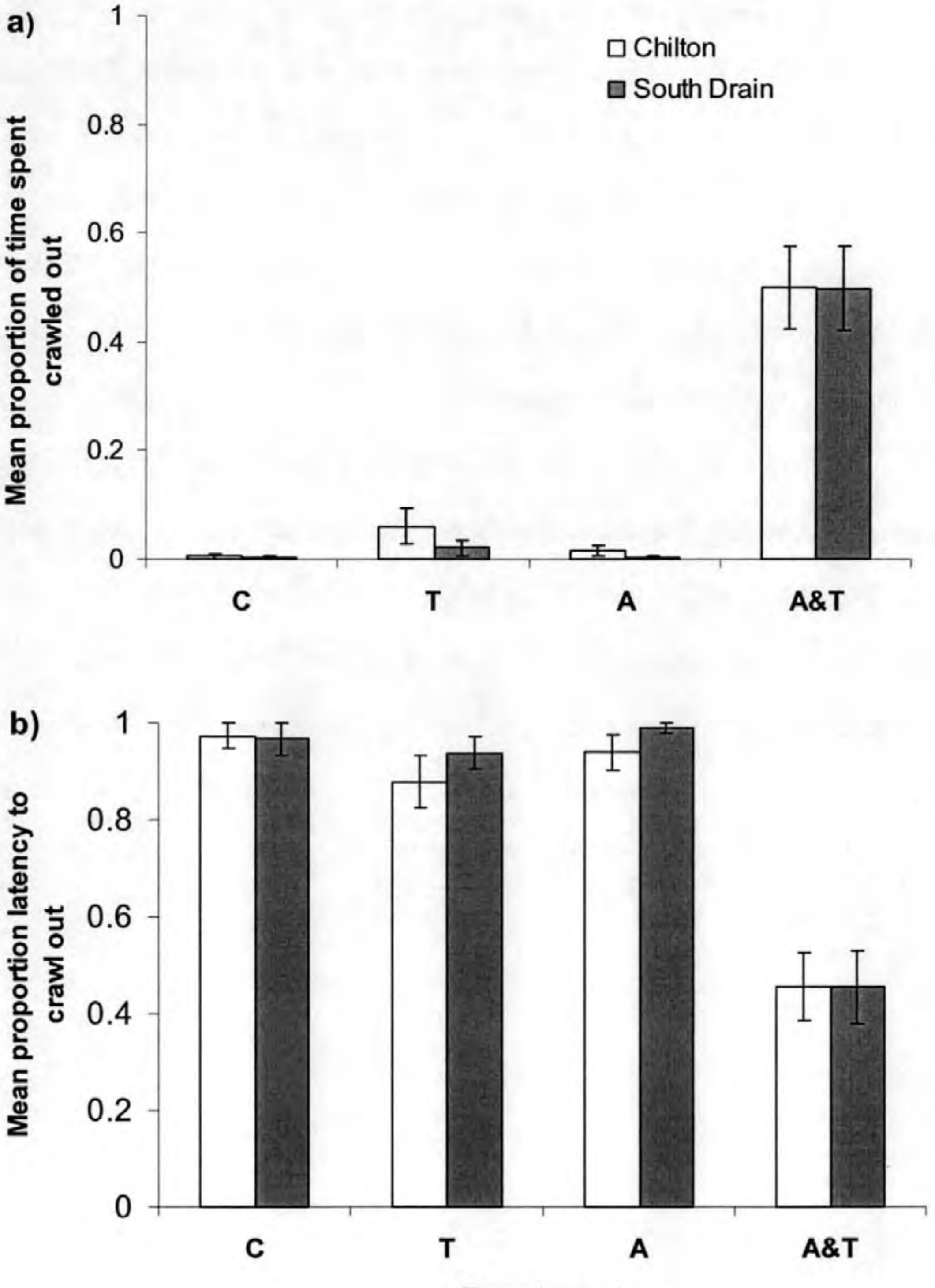

Figure 4.1: **The mean (± s.e.) crawl out response of F1 *Lymnaea stagnalis* from Chilton and South Drain responding to four different treatment levels (control, C, tench cue, T, alarm cue, A and tench paired with alarm cue, A&T) showing a) proportion of time spent crawled out and b) latency to crawl out. N=24 for each treatment combination.**

Time spent crawled out following selection

As with the F1 generation the greatest proportion of time spent crawled out of the water was in response to alarm and tench cues presented together, however the direction of selection significantly affected the level of response to combined cues in both the F2 and F3 generations for both populations, with the high response selected line spending significantly longer and the low selected lines spending significantly less time crawled out of the water compared with the non-selected line, (Fig. 4.2: Table 4.2: Se*A*T: $F_{2,1104} = 4.05$, $P = 0.018$). Selection also affected the time spent crawled out in response to alarm cue alone, with the response significantly increased in the high selection line, but only in the F3 generation for both populations (Fig. 4.2: Table 4.2: Ge*Se*A: $F_{2,1044} = 4.67$, $P = 0.010$). There was no significant effect of population on the effects of generation or selection, however the South Drain population showed a significantly higher response to tench cue alone compared to the Chilton population (Fig. 4.2: Table 4.2: Po*T: $F_{1,1104} = 10.04$, $P = 0.002$).

Table 4.2: Results from 5-way ANOVA on F2 and F3 generation snails, showing time spent crawled out and latency to crawl out. Factors included are: generation (F2/F3), population (Chilton/South Drain), selection (low/random/high) alarm cue (present/absent) and tench cue (present/absent). N = 24 for all treatment combinations.

		Proportion of time			Latency		
Source	**d.f.**	**Mean square**	**F**	**P**	**Mean square**	**F**	**P**
Generation (Ge)	1	0.072	0.71	0.398	0.074	0.49	0.483
Population (Po)	1	0.481	4.76	0.029	0.298	1.98	0.160
Selection (Se)	2	4.029	39.91	<0.001	5.789	38.44	<0.001
Alarm cue (A)	1	37.388	370.40	<0.001	41.031	272.46	<0.001
Tench cue (T)	1	29.339	290.66	<0.001	34.253	227.45	<0.001
Ge*Po	1	0.017	0.16	0.685	0.149	0.99	0.320
Ge*Se	2	0.565	5.59	0.004	0.232	1.54	0.214
Ge*A	1	0.263	2.61	0.107	0.269	1.78	0.182
Ge*T	1	0.256	2.54	0.111	0.309	2.05	0.152
Po*Se	2	0.001	0.01	0.986	0.024	0.16	0.851
Po*A	1	<0.001	<0.01	0.997	0.420	2.79	0.095
Po*T	1	1.013	10.04	0.002	1.533	10.18	0.002
Se*A	2	2.266	22.44	<0.001	1.472	9.78	<0.001
Se*T	2	1.186	11.75	<0.001	0.753	5.00	0.007
A*T	1	16.246	160.95	<0.001	8.227	54.63	<0.001
Ge*Po*Se	2	0.003	0.03	0.969	0.077	0.51	0.602
Ge*Po*A	1	0.029	0.28	0.595	0.101	0.67	0.414
Ge*Po*T	1	0.028	0.28	0.600	0.009	0.06	0.805
Ge*Se*A	2	0.471	4.67	0.010	0.521	3.46	0.032
Ge*Se*T	2	0.111	1.10	0.335	0.235	1.56	0.210
Ge*A*T	1	0.175	1.73	0.189	0.133	0.88	0.348
Po*Se*A	2	0.040	0.40	0.670	0.131	0.87	0.418
Po*Se*T	2	0.060	0.60	0.552	0.305	2.02	0.133
Po*A*T	1	0.123	1.21	0.271	0.099	0.65	0.419
Se*A*T	2	0.409	4.05	0.018	0.031	0.21	0.812
Ge*Po*Se*A	2	0.096	0.95	0.387	0.515	3.42	0.033
Ge*Po*Se*T	2	0.066	0.65	0.522	0.141	0.94	0.392
Ge*Po*A*T	1	0.225	2.23	0.135	0.249	1.66	0.198
Ge*Se*A*T	2	0.022	0.22	0.807	0.042	0.28	0.758
Po*Se*A*T	2	0.095	0.94	0.390	0.190	1.26	0.284
Ge*Po*Se*A*T	2	0.013	0.12	0.883	0.203	1.34	0.261
Total	1151						

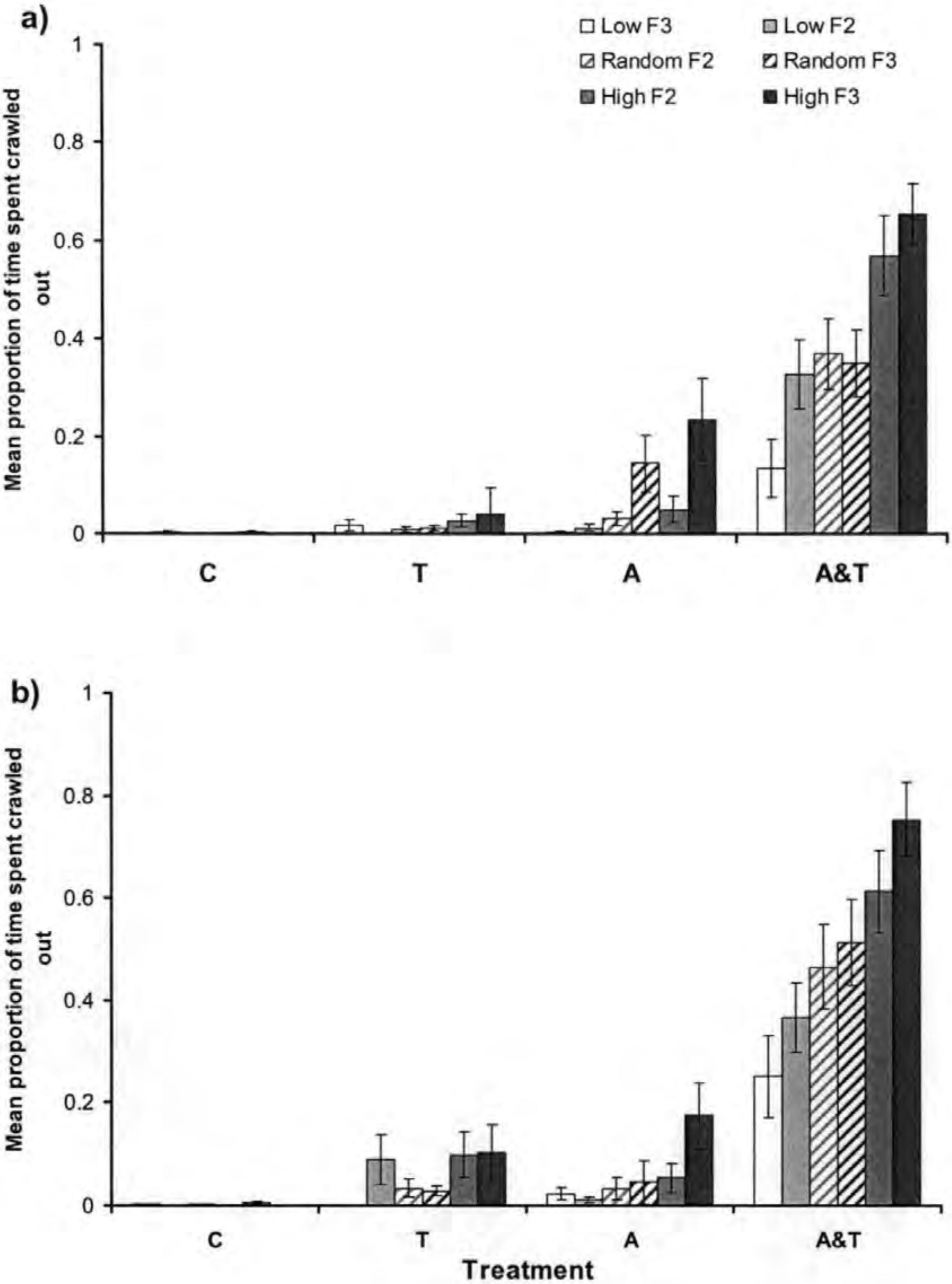

Figure 4.2: The mean (± s.e.) proportion of time spent crawled out by F2 and F3 *Lymnaea stagnalis* from low, random and high selected lines responding to four different treatment levels (control, C, tench cue, T, alarm cue, A and tench paired with alarm cue, A&T) showing a) Chilton population and b) South Drain population. N=24 for each treatment combination.

Latency to crawl out following selection

The latency to crawl out was significantly reduced in response to alarm cue following selection for individuals in the high response selected line compared to the low response selected line in the F2 generation for both populations; however this directional difference between high and low selection lines was increased in the F3 generation for the Chilton population, but was not apparent in the F3 generation for the South Drain population (Fig. 4.3: Table 4.2: Ge*Po*Se*A: $F_{2,1104} = 3.42$, $P = 0.033$). The response to tench cue was increased by selection in the high response selected lines relative to the low response selection lines, irrespective of generation or population (Fig. 4.3: Table 4.2: Se*T: $F_{2,1104} = 5.00$, $P = 0.007$).

Latency to crawl out was significantly shorter in response to combined tench kairomones and alarm cue compared to controls or either cue presented alone (Fig. 4.3: Table 4.2: A*T: $F_{1,1104} = 54.63$, $P < 0.001$). As with the time spent crawled out, the response to tench cue was greater in the South Drain population compared with the Chilton population, with significantly shorter latency to crawl out in the South Drain population compared to the Chilton population (Fig. 4.3: Table 4.2: Po*T: $F_{1,1104} = 10.18$, $P = 0.002$).

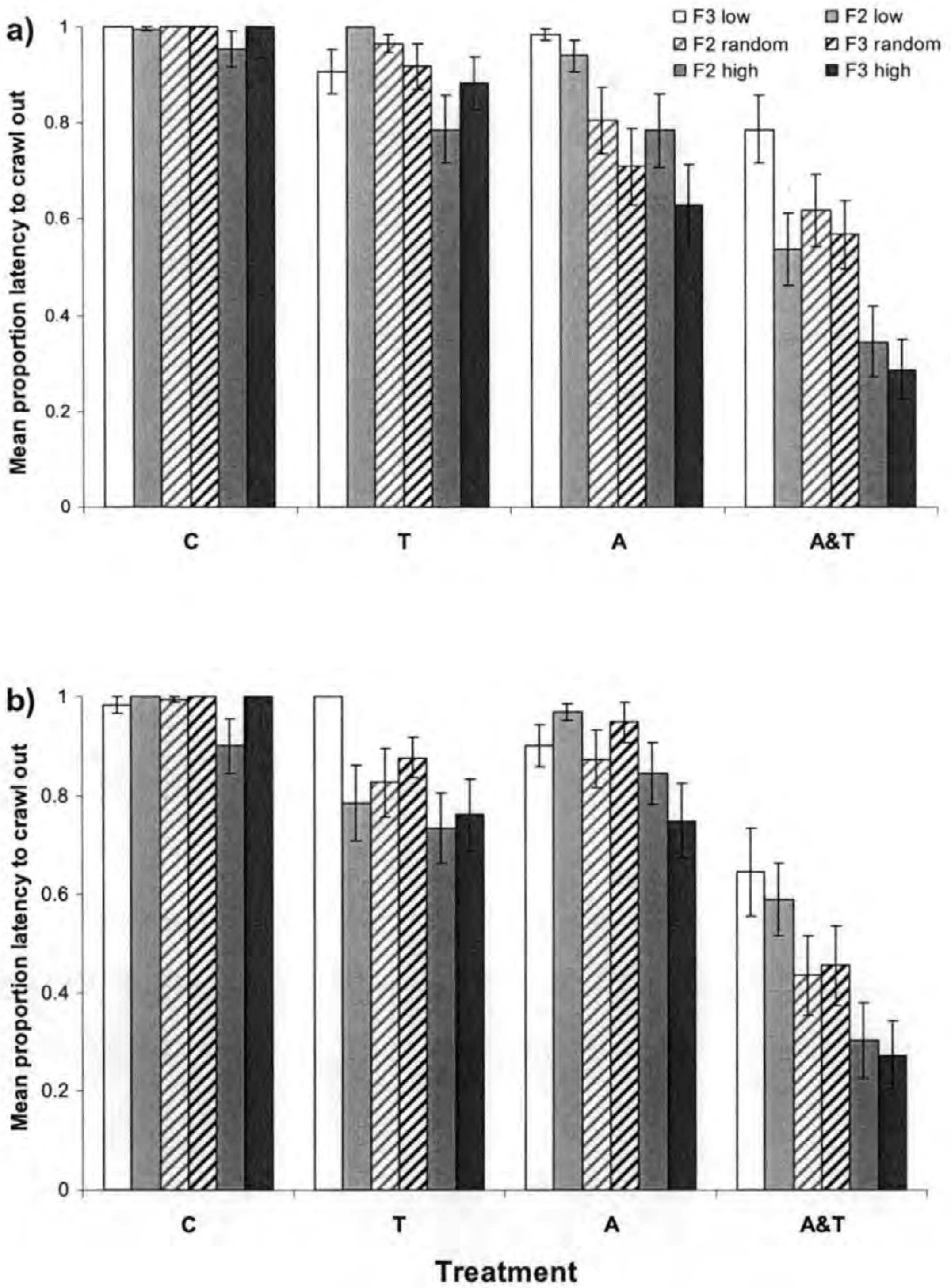

Figure 4.3: **The mean (± s.e.) proportion latency to crawl out by F2 and F3** ***Lymnaea stagnalis*** **from low, random and high selected lines responding to four different treatment levels (control, C, tench cue, T, alarm cue, A and tench paired with alarm cue, A&T) showing a) Chilton population and b) South Drain population. N=24 for each treatment combination.**

Variance and heritability of the response

The variance in response to alarm and tench cue presented together did not differ significantly following selection for either high or low response in either the time spent crawled out (Levene's test: Chilton: F = 1.353, P = 0.246; South Drain: F = 0.884, P = 0.494) or latency to crawl out (Levene's test: Chilton: F = 0.639, P = 0.670; South Drain: F = 0.822, P = 0.536). The mean heritability for crawl out behaviour did not differ significantly between the Chilton ($h^2 = 0.33 \pm 0.09$) and the South Drain ($h^2 = 0.33 \pm 0.04$) populations.

Discussion

Selection for crawl out behaviour in *Lymnaea stagnalis* in response to tench kairomones paired with alarm cues resulted in a rapid change in behaviour of the high-selected and low-selected lines within two generations. This magnitude of this change is similar to that seen in response to artificial selection on parasite resistance in another aquatic gastropod, *Biomphalaria glabrata* (Webster and Woolhouse 1999), and also to that found in several other taxa under selection for traits that may have high fitness consequences, such as: migration behaviour in the blackcap *Sylvia atricapilla* (Berthold and Helbig 1992), parasitoid resistance in *Drosophila melanogaster* larvae (Kraaijeveld et al. 2001), death feigning in Cucujiform beetles (Miyatake et al. 2004, Ohno and Miyatake 2007) and nest building in mice, *Mus domestica* (Bult and Lynch 2000). The strong response to selection found in this study suggests that the innate variation in anti-predator behaviour between *L. stagnalis* populations experiencing different predator regimes (Chapter 3: Dalesman et al. 2007b) has a fixed genetic basis rather than being a response caused by transgenerational induction (Agrawal et al. 1999).

The divergence between high and low response lines in crawl out behaviour by the F3 generation of *L.* stagnalis was similar to the divergence in innate crawl out response in natural populations of this species experiencing high and low predation risk (Chapter 3: Dalesman et al. 2007b). This response to artificial selection indicates that populations of this gastropod have the potential to respond quickly to changes in predator regime, potentially within two generations. The homogenous laboratory conditions in which selection was carried out here may have resulted in the crawl out response evolving more rapidly than it would in natural conditions. Variation in the natural environment may alter the ability of an organism to respond to selection (Wilson et al. 2006), limiting the effects of selection for a particular trait. Indeed, the evolution of natural populations often happens at a much slower rate than that potentially driven by artificial selection in the laboratory (Reznick et al. 2001).

Traits that respond to artificial selection may be negatively correlated with other fitness traits preventing the selected traits from reaching similar levels in the natural population. For example, increased duration in death feigning, shown to decrease predator vulnerability in the bean beetle, *Callosobruchus chinensis* was negatively correlated with flight ability in both artificially selected lines and in 21 natural populations (Ohno and Miyatake 2007). Similarly, responses of domesticated masu salmon (*Oncorhynchus masou*) to predator cues appears to have been selected against by selecting for fast growth, which favours responding to food but not to predators (Yamamoto and Reinhardt 2003). Negative effects may also occur under natural selection for defensive traits, for instance the loss of song in male field crickets, *Teleogryllus oceanicus*, makes them less vulnerable to parasitic flies but also prevents them from being able to attract females (Zuk et al. 2006). In the case of the behavioural traits selected for in this study, the responses to selection in the high and low lines had no apparent negative effects on fitness, however the controlled conditions in the laboratory may mask potential difficulties that *L. stagnalis* may experience in field conditions.

The *L. stagnalis* populations selected in this study came from different environments with different predator regimes (Chapter 3: Dalesman et al. 2007b), and this may account for the slight variability in their response to selection. Previous adaptation to their respective environments is likely to have resulted in genetic divergence between the populations (Bult and Lynch 2000). The differences between the populations in the response to tench cue alone also indicates that selection for the response to combined cues may be acting on different alleles controlling predator detection and anti-predator behaviour in each population (Endler et al. 2001).

Heritability estimates for crawl out behaviour in response to predation ($h^2 = 0.33$) did not differ between the populations, and this value is close to those found for other behavioural traits with high fitness-related consequences, for example the onset of migratory activity in blackcaps, *Sylvia atricapilla* (Pulido et al. 2001) and anti-predator behaviour in dumpling squid, *Euprymna tasmanica* (Sinn et al. 2006). This degree of heritability is also close to the mean for behavioural traits calculated from a review of a large number of published studies (Stirling et al. 2002). The lack of difference in heritability between populations suggests that they both retain similar underlying variability in the genes controlling crawl out behaviour.

The amount of phenotypic variation retained in the F3 generation indicates that there may still be potential for behavioural avoidance to evolve (Fisher 1930). However, if the phenotype is strongly influenced by the environment experienced during development (i.e. high levels of developmental plasticity), then a lower heritability of traits and slower response to selection may be expected (Brakefield 2003). Though this is not always the case, a positive relationship between the rate of change in a trait responding to natural selection and plasticity of that trait has been found in mosquito fish, *Gambusia affinis* (Stearns 1983). Induced morphology can also alter behaviour, showing a trade-off between energy investment in morphological and behavioural defences (Rundle et al. 2004). Longer term exposure to predator cues may induce morphological or behavioural change altering the selection strength on innate behavioural responses.

Adaptation by *L. stagnalis* to environmental conditions as demonstrated both by the variation between populations exposed to different predator regimes (Chapter 3: Dalesman et al. 2007b), and also by the strong response to selection for anti-predator behaviour shown here, would allow this species to show an innate response to predators on the first encounter. I have also shown that recent experience can provide further information about predator risk and potentially increase behavioural responses towards active predators (Chapter 2: Dalesman et al. 2006). In this way *L. stagnalis* will be able to fine-tune innate responses to predator recognition developed through selection to match the current predator activity in their environment.

CHAPTER 5

Conservation of induced anti-predator responses following experimental selection

Dalesman, S., Rundle, S. D. & Cotton, P. A. In Prep. Plastic snails: how induced responses may counteract the effects of selection.

Abstract

The way in which an organism responds to environmental stimuli may be controlled by a fixed adaptive (constitutive) response or by a plastic response induced by prior experience. The aquatic gastropod *Lymnaea stagnalis* has been shown to have constitutive behavioural responses towards cues from a predatory fish, *Tinca tinca*, which respond to artificial selection; however it has not yet been tested whether anti-predator behaviour also has induced elements. Here I exposed *L. stagnalis* from three experimental selection lines to predator cues from *T. tinca* added every four days from when the egg masses were layed. Juvenile snails that came from lines exhibiting low levels of constitutive anti-predator behaviour respond to exposure to fish cues by increasing anti-predator behaviour to the same level as that seen in lines selected for high constitutive responses. However, high response snails showed no significant change in anti-predator response following developmental exposure to fish cues, suggesting that there may be a 'ceiling effect', potentially a physiological limitation, dictating the maximum level of crawl-out behaviour *L. stagnalis* can show. The results are discussed in terms of the ability of *L. stagnalis* to cope with changes in predator regime.

Introduction

Predation imposes high fitness costs, and as such prey taxa are under strong selection to evolve effective anti-predator traits (Lima and Dill 1990, Lass and Spaak 2003, Benard 2004). These traits may be constitutive, so they are expressed whether populations of prey species experience the predator or not (Lively et al. 2000). Alternatively, prey may exhibit induced responses (Tollrian and Harvell 1999), a form of phenotypic plasticity, where the expression of the genotype varies depending on the environmental conditions (Bradshaw 1965). A meta-analysis of data from studies into the impact of predators on prey populations demonstrated that more than 50% of the impact on prey-demography is due to induced defences, such as reduction of activity and feeding time, rather than direct effects from consumption (Preisser et al. 2005). Genetic variation for plasticity exists (Stinchcombe et al. 2004), and plastic responses can be selected to alter both the mean response within a particular environment and also the degree of plasticity in response to alternate environments (Scheiner 1993, 2002, Garland and Kelly 2006).

Plasticity is thought to be favoured by selection in variable environments (Kawecki and Ebert 2004, Zhang 2006), and selection for greater trait plasticity can occur rapidly, for example in the great tit, *Parus major*, plasticity in laying time has increased significantly in response to variability in the timing of food availability (Nussey et al. 2005). In this case the increased plasticity appears to be correlated with an innate earlier laying date, however it is still not clear whether selection for plasticity is under the same control as selection for the mean of the trait, or is selected for separately as a trait in its own right (Via et al. 1995). What is clear is that the same traits can be selected both for the mean response, and also for the degree of plasticity about that mean as expressed in different environments (Noach et al. 1997, Sexton et al. 2002). Plastic traits in themselves may, potentially, have more than one heritable component, in both the magnitude of the response as shown in the above examples, and on triggers for the induced response to occur (Scheiner and Lyman 1991). Selection for recognition of the cues that induce a plastic response has been found. *Rana aurora*, for example, evolved to recognise the invasive predatory bullfrog, *R. catesbeiana* in less than 70 years (Kiesecker and Blaustein 1997), and *Gammarus pulex* adapt to respond with induced defences towards predatory fish when co-existing with them (Åbjörnsson et al. 2004).

Behavioural responses tend to have a very short lag time between the expression of one phenotype and the next, and could therefore be very flexible in how they are expressed (Gabriel et al. 2005). Selection may favour behavioural defence mechanisms where there are fitness benefits to responding rapidly to a predation threat. Innate behavioural responses may evolve where the correct response on first encountering a novel predatory threat can have high fitness consequences, whereas plastic responses may evolve where information gathered allows the response to be fine-tuned to environmental variation. Plasticity can occur during development, with the phenotype expressed in adulthood resulting from environmental cues earlier in life (Relyea 2001, Alvarez and Nicieza 2002) or environmental cues may effect the developing organism at any stage of development (Laurila and Kujasalo 1999, Laforsch and Tollrian 2004, Griffith and Sultan 2006), and demonstrate reversibility (Relyea 2003).

Theoretically, directional selection will favour plastic traits which alter phenotype in the same direction as selection on the mean (Garland and Kelly 2006). However, this is not always the case, selection on mean traits values may be correlated with plasticity, however the direction of the relationship may differ between selection lines (Scheiner and Lyman 1991). Traits under strong selection on the mean response can also exhibit the greatest degree of plasticity (Stearns 1983, Van Buskirk and Relyea 1998), though plasticity for a trait can evolve independently of the population mean for that trait (Garland and Kelly 2006).

Plasticity may confer advantage to organisms that have to deal with changes in their environment, or potentially enable them to survive in a novel environment (Parsons and Robinson 2006, Nussey et al. 2007). The ability to respond plastically to predatory threats may reduce the negative impact on fitness on encountering a novel predator, and therefore allow the animal to adapt further to increase fitness (Latta et al. 2007). However, there is also an increasing body of evidence that plasticity in a trait can also incur fitness costs (DeWitt 1998, Agrawal et al. 2002, Merilä et al. 2004, Stinchcombe et al. 2004, Teuschl et al. 2007), and may also lead to incorrect assessment of predation risk from organisms not previously encountered (Langerhans and DeWitt 2002, Schlaepfer et al. 2005).

The aquatic gastropod *Lymnaea stagnalis* is able to respond rapidly to artificial selection for anti-predator behaviour, producing levels of divergence found in innate behaviour between high and low risk environments in just two generations (Chapters 3&4: Dalesman et al. 2007b, in review). *Lymnaea stagnalis* is also able to alter perceived risk from a predator based on cues that could indicate the foraging activity of the predator (Chapter 2: Dalesman et al. 2006). It has also been shown that prolonged exposure of *L. stagnalis* to predatory fish kairomones can induce changes in shell morphology, though this is dependent on calcium availability (Rundle et al. 2004). Such morphological responses may result in trait compensation, where individuals that show strong morphological defences reduce their behavioural response (Rundle and Brönmark 2001, Cotton et al. 2004, Rundle et al. 2004), or alternatively traits may co-vary, such that prey which show strong induced morphological responses also demonstrate increased anti-predator behaviour (DeWitt et al. 1999).

This study utilised F3 generation snails selected for high and low levels of constitutive anti-predator behaviour (Chapter 4: Dalesman et al. in review), to test whether the behaviour selected for is plastic under different environmental conditions. In this case I varied the developmental conditions by growing snails in the presence or absence of fish kairomones from a known gastropod predator, *Tinca tinca* (Brönmark 1994). I investigated whether plastic behavioural responses are linked to selection on the mean innate response. I predicted that plasticity in the high response selection lines would be higher relative to the low response selection line due to a generalised increased responsiveness to predatory fish kairomone (Ruther et al. 2002).

Methods

Great pond snails, *Lymnaea stagnalis* from two source populations on the Somerset Levels, U.K., South Drain (51.18N 2.88W) and Chilton Moor (51.19N 2.88W) were selected for either high or low behavioural responses to fish predation cues (fish kairomones plus conspecific alarm cues) over three generations in the laboratory, and a randomly selected line was also bred from each population to control for effects of laboratory rearing (See Chapter 4: Dalesman et al. in review for details of selection criteria). In the laboratory, adults were kept in aquaria with 4 l of aerated artificial pond water (ASTM 1980) with 90 mgl^{-1} [Ca^{2+}] (Rundle et al. 2004) at 20±1°C under a 12:12 light:dark cycle, and fed on Iceberg lettuce and spinach.

Exposure protocol

F3 egg masses were collected from F2 adult tanks on the day they were laid and transferred into individual tanks with 1l of artificial pond water maintained at 20±1°C under a 12:12 light:dark cycle. A total of 3 tanks per population/selection line combination were used for the predator exposure treatment (fish kairomones) and 3 tanks for each of the controls (artificial pond water). Fish kairomone was produced by placing 3 tench (10±1cm) in 4 l of water for one hour. Kairomone water or control was then added at a 10% concentration to each of the tanks every 4 days. On hatching the number in each tank was reduced to 20 individuals to control density and they were maintained in otherwise analogous conditions to the F2 adults except that kairomone/control addition continued every 4 days in treatment tanks until snails reached 6±0.5mm, at which point they were used for behavioural trials.

Behavioural trials

The behavioural trials and cue production followed methodology outlined in detail in Chapter 2, with 15 individuals exposed to each of treatment: i) control (artificial pond water alone), ii) alarm cue (crushed conspecific snail), iii) tench kairomone or iv) tench kairomone plus alarm cue. The position of each snail in individual behavioural chambers was recorded for 2 hours following cue addition and crawl-out behaviour analysed as the main anti-predator response of *L. stagnalis* to fish predation threat (Chapter 2: Dalesman et al. 2006).

Data Analysis

Data were analysed as arcsine-square root proportions (Sokal and Rohlf 1995) of time spent crawled out and latency to crawl out in a 5-way ANOVA in GMAV (Institute of Marine Ecology, University of Sydney, Sydney, Australia) using population origin (Po:

Chilton /South Drain), selection line (Se: low/random/high), kairomone exposure during development (Ex: present/absent), alarm cue addition during behavioural trials (A: present/absent) and tench kairomone addition during behavioural trials (T: present/absent) as factors. Post-hoc analyses of pair-wise differences were carried out using Student-Newman-Keuls (SNK) tests in GMAV.

Results

There were no significant differences between the two populations in any of the treatment combinations tested (Table 5.1). Time spent crawled out of the water was strongest in all cases in response to tench kairomones paired with alarm cue; in the group that had not been exposed to tench kairomones during development the response to combined cues differed significantly depending on which selection line the snails came from such that strength of response high selection line > random line > low selection line. However, following exposure to tench kairomones during development, the effect of selection on the response to combined cues was eliminated, and all selection lines responded at the high response level (Fig. 5.1: Table 5.1: Se*Ex*A*T: $F_{2,672} = 3.89$, $P = 0.021$; SNK: $P < 0.01$). Exposure to tench kairomones during development also significantly increased the response to alarm cue alone in both the randomly selected group and the high response selection line (Fig. 5.1: Table 5.1: SNK: $P < 0.05$).

Latency to crawl out was shortest when snails were exposed to tench kairomones paired with alarm cue irrespective of selection line or exposure during development (Fig. 5.2: Table 5.1: A*T: $F_{1,672} = 52.34$, $P < 0.001$; SNK: $P < 0.01$), though latency was also significantly reduced relative to controls in response to either cue presented alone

(SNK: $P < 0.01$). Selection for high response to predation and exposure to tench kairomones during development both significantly reduced the latency to crawl out (Fig. 5.2: Table 5.1: Se main effect: $F_{2,672} = 19.21$, $P < 0.001$; Ex main effect: $F_{1,672} = 11.92$, $P < 0.001$).

Table 5.1: Results from 5-way ANOVA, showing time spent crawled out and latency to crawl out. Factors included are: population (Chilton/South Drain), selection (low/random/high), exposure to tench kairomones during development (present/absent), alarm cue during behavioural trial (present/absent) and tench kairomone during behavioural trial (present/absent). N = 15 for all treatment combinations.

		Proportion of time			**Latency**		
Source	**d.f.**	**Mean square**	**F**	**P**	**Mean square**	**F**	**P**
Population (Po)	1	0.160	1.09	0.298	0.004	0.03	0.874
Selection line (Se)	2	2.547	17.24	<0.001	3.337	19.21	<0.001
Developmental exposure (Ex)	1	2.369	16.04	<0.001	2.072	11.92	<0.001
Alarm cue (A)	1	32.913	222.84	<0.001	27.350	157.43	<0.001
Tench kairomones (T)	1	30.653	207.54	<0.001	26.741	153.92	<0.001
Po*Se	2	0.029	0.19	0.824	0.068	0.39	0.675
Po*Ex	1	0.073	0.49	0.483	0.199	1.15	0.285
Po*A	1	0.043	0.29	0.589	0.050	0.29	0.592
Po*T	1	0.178	1.21	0.272	0.017	0.10	0.755
Se*Ex	2	0.121	0.82	0.441	0.116	0.67	0.512
Se*A	2	0.429	2.91	0.055	0.207	1.19	0.304
Se*T	2	0.454	3.07	0.047	0.155	0.89	0.411
Ex*A	1	1.677	11.35	0.001	0.532	3.06	0.081
Ex*T	1	0.389	2.63	0.105	0.320	1.84	0.175
A*T	1	16.757	113.45	<0.001	9.093	52.34	<0.001
Po*Se*Ex	2	0.130	0.88	0.415	0.452	2.60	0.075
Po*Se*A	2	0.083	0.56	0.571	0.145	0.83	0.435
Po*Se*T	2	0.017	0.12	0.891	0.011	0.07	0.936
Po*Ex*A	1	<0.001	<0.01	0.999	0.045	0.26	0.610
Po*Ex*T	1	0.111	0.75	0.387	0.072	0.41	0.521
Po*A*T	1	0.053	0.36	0.551	0.036	0.20	0.652
Se*Ex*A	2	0.126	0.85	0.428	0.068	0.39	0.678
Se*Ex*T	2	0.532	3.60	0.028	0.346	1.99	0.138
Se*A*T	2	0.094	0.64	0.530	0.502	2.89	0.056
Ex*A*T	1	0.194	1.31	0.252	0.004	0.02	0.875
Po*Se*Ex*A	2	0.129	0.87	0.419	0.034	0.20	0.822
Po*Se*Ex*T	2	0.310	2.10	0.123	0.303	1.74	0.176
Po*Se*A*T	2	0.311	2.11	0.122	0.287	1.65	0.193
Po*Ex*A*T	1	0.004	0.03	0.870	0.048	0.28	0.599
Se*Ex*A*T	2	0.574	3.89	0.021	0.452	2.60	0.075
Po*Se*Ex*A*T	2	0.035	0.24	0.787	0.017	0.10	0.908
Total	719						

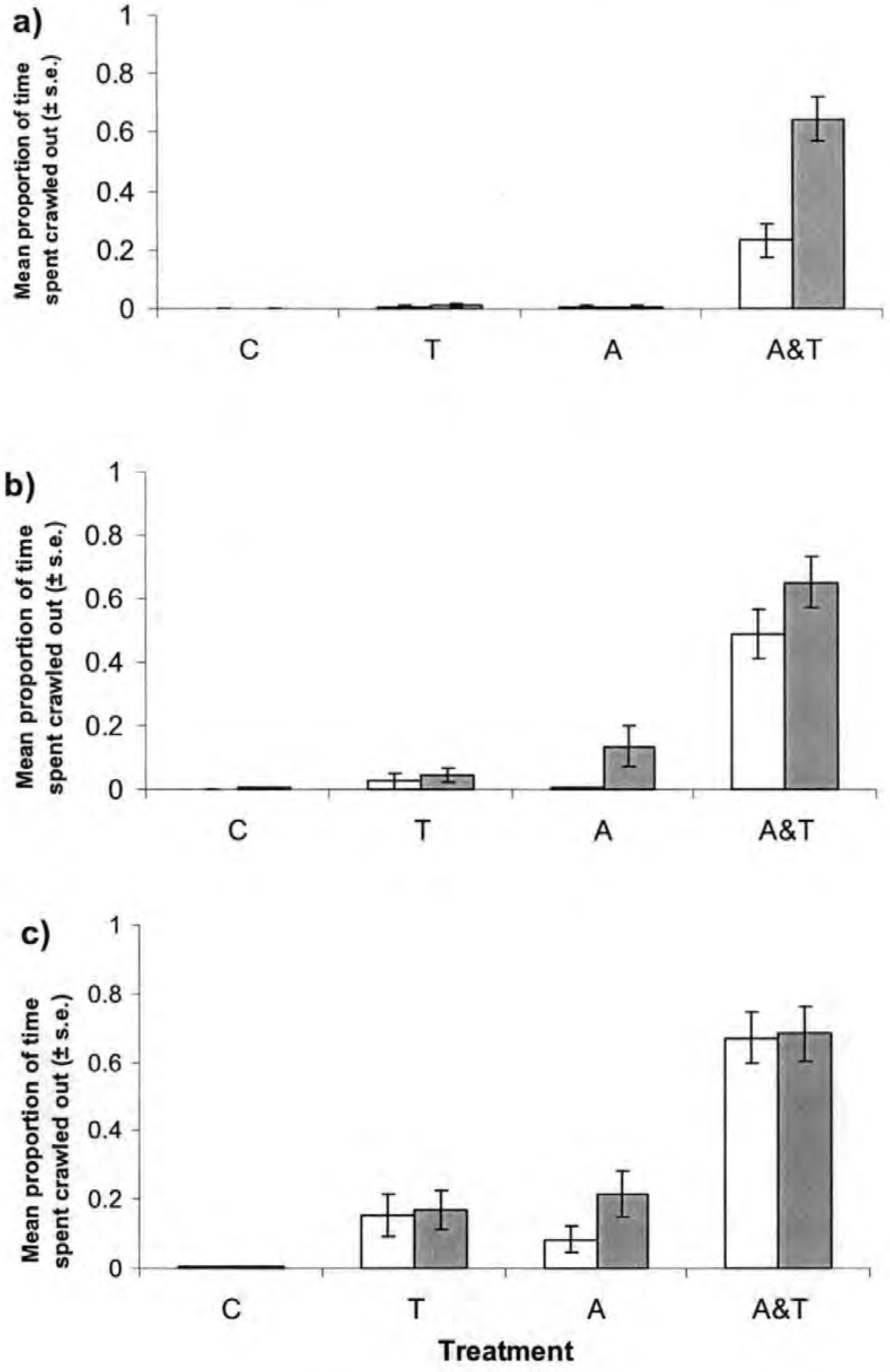

Figure 5.1: Proportion of time spent crawled out of the water in response to 4 treatments following exposure to either control (clear columns) or tench cue (shaded columns) during development following selection for a) low, b) random or c) high response to predation cues.

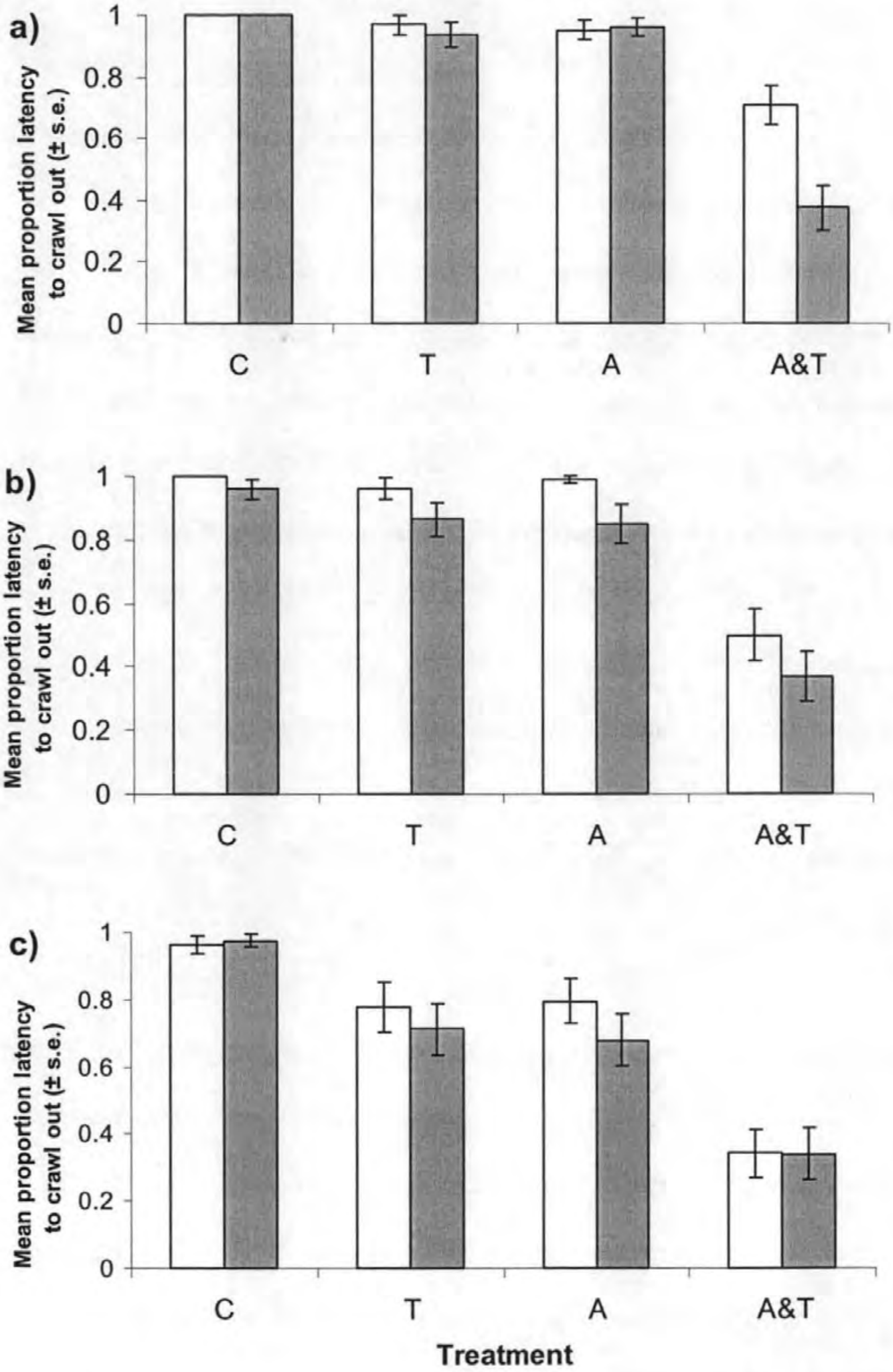

Figure 5.2: Latency to crawl out in response to 4 treatments following exposure to either control (clear columns) or tench cue (shaded columns) during development following selection for a) low, b) random or c) high response to predation cues.

Discussion

Lymnaea stagnalis, in both the low avoidance response and randomly selected lines demonstrated plasticity to exposure to tench kairomone during development. The magnitude of the induced effect was negatively correlated with the innate behavioural response, such that there was a large induced behavioural response in the low selection line and no such induced response in the high selection line. Genes controlling plasticity in behaviour may be linked directly to those controlling the innate response, such that genes coding for a low innate response are linked to those producing a large plastic response and vice versa (Via 1993, Via et al. 1995). Selection for body size at metamorphosis in the yellow dung fly, *Scathophaga stercoraria*, correlated with changes in plasticity, where large selected individuals showed induced reductions in metamorphic body size under low food conditions, but small selected lines did not, indicating that selection for large size may also have selected for increased plasticity (Teuschl et al. 2007).

Alternatively the plastic response may not be linked to the innate response. Instead all the selection lines in this study could have genetic potential to show induced responses to developmental exposure to tench kairomones, but a 'ceiling effect' limits the maximum level of crawl out response seen in the population (Garland and Kelly 2006). If this is the case then the high response selection line may mask any induced response to environmental cues by having an equally high constitutive behavioural response. The ceiling effect may be caused by a physiological limitation on the time that *L. stagnalis* can spend crawled out, or may be related to some underlying mechanism that is associated with crawl-out behaviour. The ceiling effect has been proposed as a limiting the degree of plasticity of size at metamorphosis in *Rana temporaria* responding to pond drying conditions (Merilä et al. 2004). It was also proposed as an alternative explanation as to why no plastic size reduction was seen in the small selected lines of *Scathophaga stercorari* (Teuschl et al. 2007). In both cases it was proposed that there is a physiological minimum size at which metamorphosis can occur successfully.

The level of plasticity shown in the populations used for this study may reflect past micro-evolution resulting from the conditions in which they are found. Migration between populations found in distinct habitats, in this case with different predator regimes, may favour the evolution of plasticity in traits responding to the differences between the habitats (Kingsolver et al. 2002). Plastic response in this case may reduce the effect of natural selection in the field in individuals moving into fish habitats by reducing selection pressure (Fordyce 2006), such that if eggs are laid in a habitat with fish predators offspring will produce higher levels of anti-predator behaviour. It has been proposed that the ability to show plastic responses may contribute towards the invasive ability of a species (Parsons and Robinson 2006, Richards et al. 2006). Gastropod species within the family Lymnaeidae vary considerably in their ability to colonise different habitats, potentially in part due to slow responses to different predator regimes in each environment. Species comparisons within this clade on how responses to environmental cues during development affect behavioural responses to potential predatory species may shed light on why some lymnaeid species are common in several different habitats (e.g. *Lymnaea stagnalis* and *Radix balthica*), whilst others appear more specialist and are restricted in the habitat type they can colonise (e.g. *Radix auricularia* and *Omphiscola glabra*).

Costs of plasticity can include maintaining sensory and regulatory systems to produce a plastic response, the need to obtain information from a potentially risky environment to produce the correct response or constraints on adaptation of an optimal phenotype (DeWitt 1998, Relyea 2002a, Merilä et al. 2004). Lag times may occur between variation in the environment and expression of a phenotype, resulting in a costly mis-match (Padilla and Adolph 1996, Gabriel 2006, Hoverman and Relyea 2007). There may also be costs associated with inappropriate responses to novel species (Langerhans and DeWitt 2002, Schlaepfer et al. 2005). These costs are thought to result in selection against plasticity in traits in populations where it is not required. The lack of difference between populations found here indicates that this selection against plasticity is not occurring under natural conditions as the populations come from high (South Drain) and low (Chilton) risk environments with respect to fish predation (Chapter 3: Dalesman et al. 2007b). The plastic response is retained at a high level in populations not exposed to predators, possibly as selection against a plastic response in the absence of predators is weak, and the high level of response seen in crawl out behaviour here may be restricted from increasing any further by other physiological limitations unassociated with predator recognition or the response.

The increase in behavioural avoidance following exposure to tench kairomones contradicts the findings of Rundle *et al.* (2004) who found that 5mm *L. stagnalis* reduced their behavioural avoidance of fish kairomones following exposure to kairomones during development. They proposed that this was due to an increase in shell thickness providing increased morphological protection resulting in trait compensation. This contradiction may be due to a difference in kairomone exposure between the two studies: in Rundle *et al.* (2004) exposure was constant, whereas in this study kairomone addition only occurred every four days. Alternatively it may be due to exposing *L. stagnalis* to predator cues from an earlier stage of development. Morphological defences are potentially more costly than behavioural defences (DeWitt 1998, Trussell and

Nicklin 2002), and may also incur further costs if they lag behind environmental change (Padilla and Adolph 1996, Gabriel et al. 2005, Hoverman and Relyea 2007), as such they may only be induced under constant predation threat. Cue strength decays exponentially over time (Turner and Montgomery 2003), therefore the snails in this study will not have been under constant exposure to kairomones. Although laboratory rearing has not affected the behavioural responses between F1 and F3 generations, there is also the possibility that the morphological response, or the relationship between morphology and behaviour, has been affected by rearing successive generations under controlled conditions.

These results indicate that selection for the constitutive elements of anti-predator behaviour is under a different control mechanism to the induced response from prolonged exposure to tench kairomones during development. To untangle the relationship between the level of innate response and the degree of plasticity in the response shown in *L. stagnalis* crawl out behaviour, further work is required where selection for plasticity rather than for the value of the constitutive response is carried out. The relative ease with which selection for behavioural traits can be carried out, and the potential for plastic responses in morphology (Rundle et al. 2004) as well as behaviour (Chapter 2: Dalesman et al. 2006), make *L. stagnalis* and ideal model to test the relationship between plastic and constitutive responses.

I have previously demonstrated that exposure to predation cues increased the perceived risk to tench kairomones alone (Chapter 2: Dalesman et al. 2006). Here I demonstrate that prolonged exposure to tench kairomones during development can increase the anti-predator behaviour of a population, generally showing a low response to predation cues. Combined with differences in the innate response between populations (Chapter 3:

Dalesman et al. 2007b), which is due to genetic adaptation (Chapter 4: Dalesman et al. in review), these data indicate that *L. stagnalis* has evolved to utilise all available information to identify potential predators in their environment and respond appropriately.

CHAPTER 6

Phylogenetic relatedness and ecological interactions determine anti-predator behaviour.

Dalesman, S., Rundle, S. D., Bilton, D. T. & Cotton, P. A. 2007. Phylogenetic relatedness and ecological interactions determine anti-predator behaviour. *Ecology*. In Press.

Abstract

Interspecific recognition of alarm cues among guild members through 'eavesdropping' may allow prey to fine-tune anti-predator responses. This process may be linked to taxonomic relatedness, but might also be influenced by local adaptation to recognize alarm cues from sympatric species. I tested this hypothesis using anti-predator responses of *Lymnaea stagnalis* (L.) to alarm cues from damaged conspecific and ten heterospecific gastropod species. As predicted, the magnitude of anti-predator response decreased significantly with increasing phylogenetic distance, but increased when species were naturally sympatric (defined as species co-habiting in the same water body) with the source population of *L. stagnalis*. The responses to sympatric species were higher overall, and the relationship between genetic distance and alarm cue response was stronger when tested with sympatric species. This is the first study to demonstrate that population sympatry influences innate anti-predator responses to alarm cues from intraguild members and suggests that responses based on phylogenetic relationships can be modified through local adaptation. Such adaptation to heterospecific alarm cues suggests that species could be at a disadvantage when they encounter novel intraguild members resulting from species invasion or range expansion due to a reduction in the presence of reliable information about predation risk.

Introduction

Biological signals produced by organisms are generally intended to communicate only with conspecific individuals conferring benefit to the signaller, but may be intercepted by heterospecific individuals through 'eavesdropping' (Stowe et al. 1995, Bradbury and Vehrencamp 1998). Eavesdropping implies no benefit to the signaller, but may be of benefit to the receiver, for example in locating resources or identifying potential competitors, and has been found to be important in a wide variety of taxa (Catchpole and Leisler 1986, Stowe et al. 1995, Taga and Bassler 2003, Symonds and Wertheim 2005, Runyon et al. 2006). Eavesdropping on alarm cues from heterospecific intraguild members has been found in several species and may be used to provide additional information about potential predation risk (Stenzler and Atema 1977, Mirza and Chivers 2001a, Hazlett and McLay 2005, Schoeppner and Relyea 2005).

In communication systems where it is beneficial to both signaller and receiver to respond to related heterospecifics, phylogenetic relationships generally appear to explain the patterns observed, for example, the similarity of chemical aggregation signals in closely related *Drosophila* spp. (Symonds and Wertheim 2005) or attraction of *Aplysia* spp. to heterospecifics during egg laying (Cummins et al. 2005). In these cases, there is a benefit to all individuals to oviposit in the same site, irrespective of their species. The interaction between evolutionary history and local adaptation in shaping interspecific communication has also been investigated in relation to sexual signals, where it has been shown that differences between signalling systems are more pronounced in sympatric species pairs (Ryan and Rand 1995, Symonds and Elgar 2004). Phylogeny is not a good predictor of the degree of divergence seen between sexual signals when species are found sympatrically, though exceptions have been found (Smith and Florentino 2004). The importance of sympatry in shaping sexual signals suggests that local adaptation to avoid heterospecific interactions is acting on this signalling system, and in many cases counteracting the effects of phylogenetic relatedness.

In predator-prey systems, recognition of alarm cues from closely related species is not considered communication as there is no benefit to the signaller (Bradbury and Vehrencamp 1998), however it may be of benefit to the receiver to assess predation risk accurately and, hence, may reduce the high fitness costs associated with predation (Lima and Dill 1990). There is considerable evidence that alarm cues are conserved within phylogenetic groups (Pfeiffer 1977), with responses to alarm cues from closely related species generally being stronger than from those more distantly related (Snyder 1967, Stenzler and Atema 1977, Brown et al. 2003, Vilhunen and Hirvonen 2003), though the nature of the relationship between phylogenetic distance and response has only been investigated for the tadpole *Hyla versicolor* (Schoeppner and Relyea 2005). Snyder (1967) proposed that responses to alarm cues from heterospecific species sharing a common predator would be advantageous to a prey species regardless of taxonomic relationships. This theory would suggest that the effect of phylogenetic relatedness might be modified via the effects of sympatry or allopatry. There have been several studies which suggest that sympatry may modify responses to alarm cues from heterospecific species, however, they either used wild caught individuals so could not rule out the effects of experience (Stenzler and Atema 1977, Chivers et al. 1997, Rochette and Dill 2000, Sullivan et al. 2003, Hazlett and McLay 2005) or used too few species to investigate any interaction with the effects of phylogenetic relatedness (Laforsch et al. 2006).

In aquatic systems chemical cues often provide the most reliable source of information about a predator where other information, such as visual cues, may be impaired by poor visibility (Dodson et al. 1994). Information from predator diet or injured conspecifics can be used to assess current predator feeding activity and hence associated risk from a potential predator (Chapter 2: Dalesman et al. 2006), or to learn to identify a previously unrecognized predation threat (Chivers and Smith 1998). With strong selective forces operating on prey species to respond correctly to predation threat it seems probable that the relationship between phylogenetic distance of intraguild members from a responder and anti-predator response to alarms cues will be modified by local adaptation.

Here I investigated the innate response of a freshwater gastropod, *Lymnaea stagnalis* (L.) to alarm cues from heterospecific prey guild members, presented with cues from a fish, (tench, *Tinca tinca* L.) known to feed on gastropods (Brönmark 1994). I used ten heterospecific freshwater gastropods at differing phylogenetic distance from *L. stagnalis*, five of which are found sympatrically to the source population of *L. stagnalis* used during behavioural trials, and five of which are allopatrically distributed. In this case species were considered allopatric if they were not found in the same body of water (South Drain) as the *L. stagnalis* population (Dalesman, unpublished data), allopatric species in some cases came from a site less than 1km away. I tested the following hypotheses: (1) that the innate anti-predator response of *L. stagnalis* to alarm cue, paired with predatory fish cues, would decrease with increasing phylogenetic distance of the heterospecific snail species used to produce alarm cue; and (2) that the response to alarm cue from species found sympatrically to the *L. stagnalis* population would induce stronger anti-predator behaviour than alarm cue from species found allopatrically distributed relative to the *L. stagnalis* population.

Methods

Study Organisms

Adult *L. stagnalis* used to establish the snail culture for behavioural trials were collected from South Drain, a drainage canal on the Somerset Levels, U.K. (51.18N 2.88W) in September 2005. Repeated field sampling between June 2004 and June 2006 identified which gastropod species were present at the South Drain site. In the laboratory, adults were kept in aquaria with 4 l of aerated artificial pond water (ASTM 1980) with 90 mgl^{-1} [Ca2+] (Rundle et al. 2004). They were maintained at 15±1 °C under a 12:12 light:dark cycle, and fed on Iceberg lettuce and spinach. To minimize the influence of adult experience on juvenile development and behaviour, the F1 snails used during behavioural trials were bred from egg masses laid a minimum of two months post collection and were then maintained separately in the same conditions as the adults. Heterospecific adult snails were collected from several locations (Table 6.1) to raise F1 snails that were crushed to produce alarm cue. All snails used had a standard spire height of 6±0.5mm except in the case of ramshorn species (*Planorbis planorbis* and *Planorbarius corneus*) where spiral diameter measured 6±0.5 mm.

Genetic distance between species was calculated using a 370bp section of 18S rDNA obtained from Genbank (Table 6.1). Sequences were aligned using ClustalX 1.83 (Thompson et al. 1997) and a pair-wise distance matrix calculated in TREECON 3.0 (Van de Peer and De Wachter 1993) using the Kimura two-parameter model (Kimura 1980), with the transition/ transversion ratio estimated from the data and insertions/deletions taken into account. As no 18S rDNA sequence is currently available for *Lymnaea fusca*, the sequence for *L. palustris* was used as a closely related species within the sub-genus *Stagnicola*; *Lymnaea palustris* is the only other British species found in the subgenus (Anderson 2005), and is known to be very closely related to *L. fusca* based on ITS-2 sequence data (Bargues et al. 2001).

Odour production

Predator and alarm cues were produced in the same way in all the experiments (Chapter 2: Dalesman et al. 2006). Tench, *Tinca tinca*, were used to produce predator cue as they are a known natural predator of freshwater gastropods (Brönmark 1994). The tench were fed Nutri-Flake© (Hozelock Cyprio, U.K.), which contains no mollusc extracts, and hence avoided potential dietary cues affecting the trials. Predator cue water was produced by placing three tench (length 10±1 cm) into 4 l of aerated artificial pond water for 1 hour. Alarm cue was produced by mixing three crushed snails (6±0.5 mm) into 4l of aerated artificial pond water. Predator plus alarm cue water was produced by mixing three crushed snails (6±0.5 mm) into 4 l of tench cue water. The control was the addition of aerated artificial pond water with no cues added. Crushed snails were used as opposed to feeding snails to tench to ensure that the alarm cue concentration paired with tench cue was kept at a similar level for all behavioural trials, the strong behavioural response in the paired cue treatment in a previous study suggested that *L. stagnalis* responds to alarm cue from crushed snails paired with tench cues as a predation threat (Chapter 2: Dalesman et al. 2006).

Table 6.1: Gastropod species used to produce alarm cue. Location where adult stocks were collected and Genbank accession number for the source of 18S sequence data used to calculate Kimura 2-parameter genetic distance is shown. Species found sympatrically to the *Lymnaea stagnalis* population used as responders are shown as originating from South Drain. Nomenclature follows Anderson (2005).

Order and Family	Gastropod Species	Original population location	Genbank Accession	Kimura 2-parameter distance from *L. stagnalis*
Basommatophora Lymnaeidae	*Lymnaea stagnalis* (Linnaeus, 1758)	South Drain 51.18N 2.88W	Z73984	0
Lymnaeidae	*Lymnaea fusca* (C. Pfeiffer, 1821) *	North Moor 51.07N 2.96W	Z73983	0.543
Lymnaeidae	*Omphiscola glabra* (Müller, 1774)	Bodmin Moor 50.56N 4.67W	Z73982	1.094
Lymnaeidae	*Galba truncatula* (Müller, 1774)	Clyst St Mary 50.71N 3.46W	Z73985	3.645
Lymnaeidae	*Radix auricularia* (Linnaeus, 1758)	South Drain 51.18N 2.88W	Z73980	5.265
Lymnaeidae	*Radix balthica* (Linnaeus, 1758)	South Drain 51.18N 2.88W	Z73981	5.552
Physidae	*Physa fontinalis* (Linnaeus, 1758)	North Moor 51.07N 2.96W	AY577486	8.259
Physidae	*Physella acuta* (Draparnaud, 1805)	South Drain 51.18N 2.88W	AY282600	8.561
Planorbidae	*Planorbis planorbis* (Linnaeus, 1758)	Chilton Moor 51.19N 2.89W	AY577497	9.081
Planorbidae	*Planorbarius corneus* (Linnaeus, 1758)	South Drain 51.18N 2.88W	AY577494	10.022
Mesogastropoda Hydrobidae	*Bithynia tentaculata* (Linnaeus, 1758)	South Drain 51.18N 2.88W	AF367675	12.497

* Sequence used is for *Lymnaea palustris* (Müller, 1774) which is very closely related to *Lymnaea fusca* (Bargues et al. 2001).

Behavioural Assay

Behavioural trials were carried out between January and May 2006 in the Experimental Aquatic Ecology Laboratory, University of Plymouth in conditions analogous to those in which the snails were maintained (Chapter 2: Dalesman et al. 2006). 24 individuals were used for each treatment combination, with each snail used once only, giving a total of 1056 snails. Behavioural chambers consisted of a white plastic dish, 165mm diameter x 60mm depth (A.W.Gregory & Co. Ltd., U.K.), with a longitudinally sectioned white PVC pipe, 36mm long, 30mm diameter, attached open side down to the centre using non-toxic sealant (Wickes Ultimate Sealant and Adhesive©, Wickes Building Supplies Ltd., U.K.) to provide a refuge. Twenty-four chambers were set up for each behavioural trial, and juvenile snails were acclimated to behavioural chambers for 24 hours in 630ml of artificial pond water prior to behavioural assays. 70ml of cue water was added to each chamber at the start of the behavioural assay to give a final concentration of 10% cue water, with 6 chambers having each of the four cue treatments added on each day. To avoid cross-contamination, alarm cue from a single species was used on each day, resulting in four behavioural trials in total for each snail species. The position of the chambers in which each treatment level was added was fully randomized within the 24 chambers for each behavioural trial. The position of each snail within the behavioural chambers was recorded every 5 minutes for 2 hours following cue addition. Crawl-out behaviour is the main anti-predator response of *L. stagnalis* to tench (Chapter 2: Dalesman et al. 2006), hence only results from crawl-out behaviour are presented here. Crawl-out behaviour was analyzed using two variables: the proportion of total time spent crawled out of the water; and the latency to crawl-out, calculated as the proportion of total time during the trial before crawl-out behaviour was observed (Cotton et al. 2004).

Data Analysis

Data were analyzed as an unbalanced ANCOVA design using General Linear Model (GLM) in Minitab13. Alarm cue, tench cue and environment (whether snails were found sympatrically or allopatrically) were treated as fixed factors, and pair-wise genetic distance between *L. stagnalis* and each of the species used to produce alarm cue was used as the covariate. Both time spent crawled out and latency to crawl out were calculated as proportions of total duration, and were arcsine-square root transformed prior to analysis (Sokal and Rohlf 1995). Post-hoc tests on response means versus genetic distance were carried out using a regression analysis in Minitab13.

Results

The principal anti-predator behaviour of *Lymnaea stagnalis*, crawling above the water line, was greatest in response to alarm and tench cue combined, both in the mean proportion of time spent out of the water and the mean latency to crawl out. The effects of genetic distance or sympatry/allopatry were not seen in response to the control or either cue presented alone. However, in response to combined cues (alarm and tench cue) the proportion of time spent crawled out declined significantly with genetic distance between *L. stagnalis* and the heterospecific snail. This trend was modified depending on whether the snail species used is found allopatrically or sympatrically to the responding population of *L. stagnalis*; the response to allopatric species was significantly lower than that to sympatric species, with a weaker decline with increasing genetic distance (Fig.6.1a: Table 6.2: ANCOVA: 4-way interaction: $F_{1,1040} = 4.84$, $P = 0.028$). In response to combined cues the latency to crawl out was significantly longer with increased genetic distance; this response was again modified by whether heterospecifics used to produce alarm cue were found sympatrically to the *L. stagnalis* population, with a significantly shorter latency to crawl out when crushed snails were found in sympatry (Fig. 6.1b: Table 6.2: ANCOVA: 4-way interaction: $F_{1,1040} = 3.95$, P = 0.047).

Table 6.2: Results from 4-way ANCOVA using genetic distance as the covariate. Results for proportion of total time spent crawled out and latency to crawl-out in response to genetic distance, environment (sympatry/allopatry), alarm cue and tench cue. N = 24 for all treatment combinations.

		Proportion of time			**Latency**		
Source	**d.f.**	**Mean square**	**F**	**P**	**Mean square**	**F**	**P**
Kimura 2-parameter genetic distance (Dis)	1	5.53	105.34	<0.001	7.23	74.26	<0.001
Environment (Env)	1	3.04	57.94	<0.001	3.77	38.72	<0.00
Alarm cue (Al)	1	7.19	136.75	<0.001	5.83	59.85	<0.001
Tench cue (Te)	1	15.35	292.21	<0.001	20.16	207.09	<0.001
Env*Dis	1	0.90	17.14	<0.001	1.34	13.80	<0.001
Al*Dis	1	2.15	40.85	<0.001	1.18	12.15	0.001
Te*Dis	1	4.64	88.29	<0.001	3.78	38.84	<0.001
Env*Al	1	1.30	24.80	<0.001	1.69	17.31	<0.001
Env*Te	1	2.89	54.91	<0.001	3.06	31.48	<0.001
Al*Te	1	7.92	150.79	<0.001	8.13	83.53	<0.001
Env*Al*Dis	1	0.29	5.53	0.019	0.58	5.99	0.015
Env*Te*Dis	1	0.87	16.46	<0.001	1.20	12.36	<0.001
Al*Te*Dis	1	2.39	45.42	<0.001	1.84	18.94	<0.001
Env*Al*Te	1	1.19	22.69	<0.001	1.22	12.63	<0.001
Env*Al*Te*Dis	1	0.25	4.84	0.028	0.38	3.95	0.047
Total	1055						

Post-hoc analysis of the response to combined cues (alarm and tench cue together) showed that when alarm cue was produced by sympatric species there was a highly significant relationship between genetic distance and strength of the response for both the proportion of time crawled out (Fig. 6.1a; R^2(adj) = 0.90, P = 0.003) and latency to crawl out (Fig. 6.1b; R^2(adj) = 0.94, P = 0.001). However, this relationship was not apparent when the species used to produce alarm cue were allopatric, with a weaker non-significant relationship between genetic distance and proportion of time spent crawled out (Fig. 6.1a; R^2(adj) = 0.63, P = 0.070), and no apparent relationship between genetic distance and latency to crawl out (Fig. 6.1b; R^2(adj) < 0.01, P = 0.396).

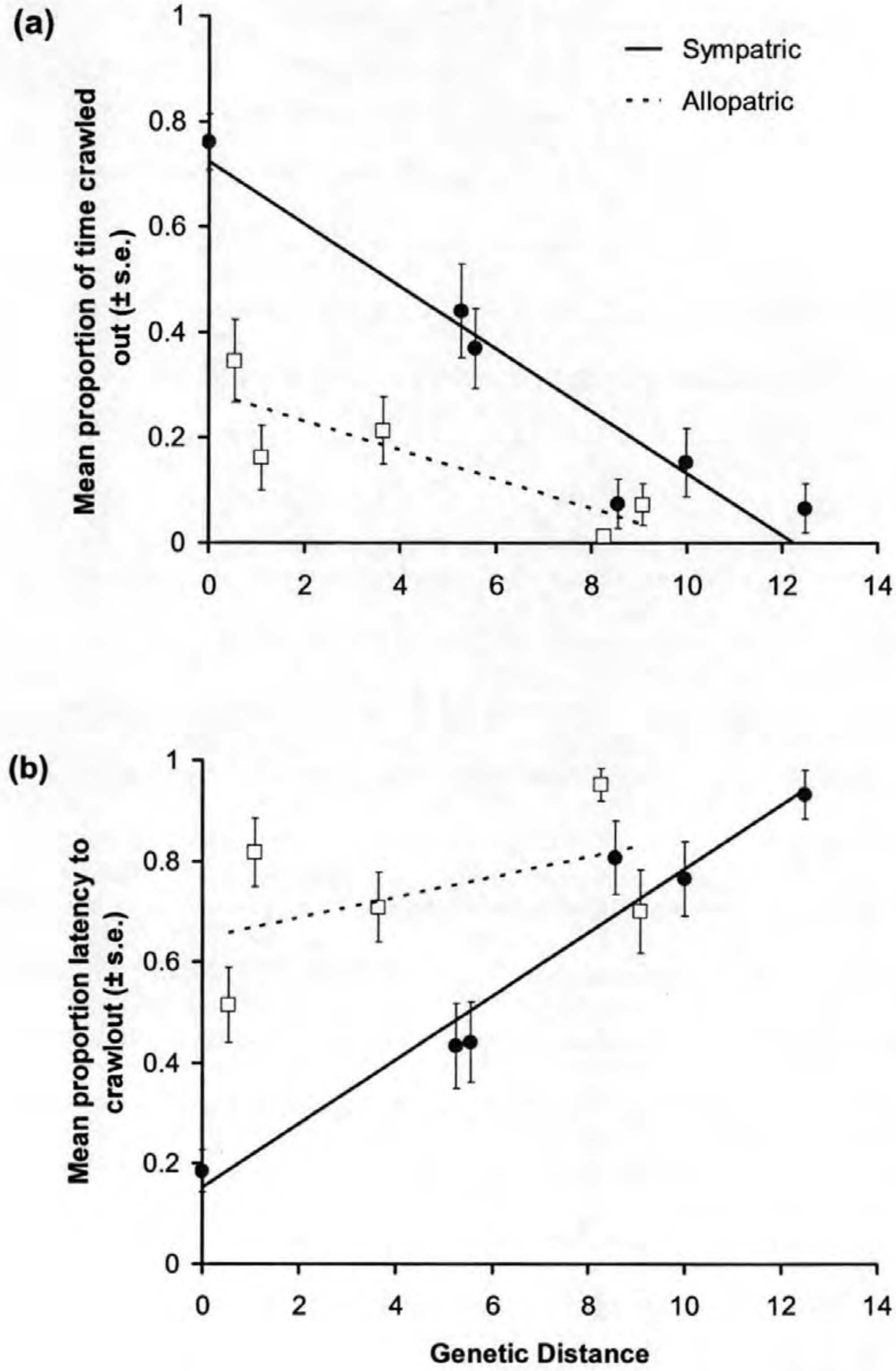

Figure 6.1: The relationship between genetic relatedness and sympatry/allopatry in the anti-predator response of *Lymnaea stagnalis* to tench and alarm cues combined. Individual symbols show the mean response (± s.e.) towards each species (● = sympatric species, □ = allopatric species), with lines indicating the trend in relationship between behaviour and Kimura 2-parameter genetic distance for (a) Proportion of time spent crawled out of the water. (b) Proportion latency to crawl out. N = 24 for each data point.

Discussion

This study demonstrates that the magnitude of innate avoidance responses shown by *Lymnaea stagnalis* to heterospecific alarm cues paired with predator cues declines with phylogenetic distance. This declining response concurs with findings on innate tadpole behaviour (*Hyla versicolor*) where timing of evolutionary divergence was compared with response level (Schoeppner and Relyea 2005). Although the relationship between genetic distance and anti-predator response of *L. stagnalis* is very strong when the responding population is found sympatrically to the species used to produce alarm cues, this relationship is weaker and not significant when considering the response to allopatric species, particularly in the latency to crawl out. This is the first evidence to demonstrate that the effect of relatedness on prey responses to heterospecific alarm cues may be modified by ecological context and may disappear if the species used are allopatric to the responding population.

The agreement between my findings for sympatric species and those of other studies (Mirza and Chivers 2001a, Schoeppner and Relyea 2005, Kelly et al. 2006) suggests that the influence of phylogenetic relatedness may indeed be widespread in chemical alarm signalling. This relationship between response and genetic distance indicates there may be a gradual mode of evolution in either the signalling chemicals or the way in which they are detected (Jovelin et al. 2003, Symonds and Wertheim 2005, Kelly et al. 2006). The lack of response by *L. stagnalis* to alarm cues presented in isolation alone concurs with previous findings for this species (Chapter 2: Dalesman et al. 2006), and may reflect the general unreliability for prey species of using alarm cues alone, as it provides no information about the nature of the threat (Chivers and Smith 1998).

The significant effect of sympatry on intensifying anti-predator response suggests that *L. stagnalis* has either conserved its response relative to allopatric species or elevated it towards sympatric species. This type of adjustment has been demonstrated previously in response to predator cues where individuals from environments with high numbers of predators show a greater innate response to predator cues than those from low risk environments (Boersma et al. 1998, Magurran 1999, Åbjörnsson et al. 2004), although this may be due to transgenerational effects as well as local adaptation (Agrawal et al. 1999). However, our study is the first where sympatry has been shown conclusively to affect the innate anti-predator response to alarm cues from heterospecifics. Previous studies that inferred evidence for an effect of sympatry did not control adequately for either the effects of experience, by using wild caught individuals (Stenzler and Atema 1977, Chivers et al. 1997, Rochette and Dill 2000, Sullivan et al. 2003, Hazlett and McLay 2005), or used too few species to draw strong conclusions (Laforsch et al. 2006). Together these factors may account for the bias towards the importance of phylogenetic relationships affecting the response to alarm cues in the current literature.

Although tench are likely to represent the main predation threat to aquatic gastropods in the site where our responding population was sourced, it is likely that *L. stagnalis* and co-habiting gastropod species will encounter other predator threats. The presence of generalist predators or specialist predators that focus on the taxonomic group including the prey species of interest, is likely to increase selection to respond to heterospecific cues, whereas specialist predators that focus on other taxonomic groups would select against costly induced responses to heterospecific cues. The degree to which a taxonomic group is represented in the predator's diet will affect the degree to which a response to heterospecific alarm cues is appropriate. For example, both crayfish, *Pacifastacus leniusculus*, and tench, *Tinca tinca*, consume a large number of gastropods, so there is a high probability that when they are actively foraging in an area all gastropod species have a high risk of predation (Brönmark 1994, Nyström et al. 2001). However, if a rainbow trout (*Oncorhynchus mykiss*) which specializes on aquatic insects, happens to consume an aquatic gastropod, it is unlikely that they will pose a significant threat to other gastropods in the vicinity (Nyström et al. 2001). This may have two effects on the response to alarm cues from heterospecifics: firstly the response may be predator specific, such that the prey species only responds to heterospecific cues when paired with cues from specific predators', secondly, the selection to respond to heterospecific species may only occur when there are predators present that predate several species within a taxonomic group.

A general reliance on heterospecific cues to indicate the level of predation risk in the environment may be detrimental if invasive intraguild species move into the habitat whose alarm cues are not recognized. In this case the responding species may under-estimate the risk of predation resulting in a reduced anti-predator response and potentially increased vulnerability to predation. Likewise, if a species colonizes a novel habitat it may encounter new intraguild members. The phylogenetic relationship between the responding species and the novel intraguild members is likely to influence the potential for recognition of alarm cues as demonstrated in this study. Adaptive responses to a novel species appear to have occurred historically in the population of *L. stagnalis* used as responders. I found that *L. stagnalis* exhibits an elevated response to the invasive gastropod *Physella acuta*. This response to a sympatric alien is elevated relative to that for a closely related native British species, *Physa fontinalis* found allopatrically distributed relative to the South Drain *L. stagnalis* population.

Differences between populations in response to alarm cues have previously been suggested to occur through learning (Brown 2003), however there is good support for genetic differentiation in response to predators (Åbjörnsson et al. 2004, Kawecki and Ebert 2004) and it would therefore seem highly likely that prey species would also show adaptive variation in response to alarm cues. It appears, in my study, that individuals from the population used as responders have modified their response towards sympatric species. As my responders were naïve lab reared snails I am able to rule out effects of experience, which have been used previously to explain strong responses to sympatric species (Brown

2003). Here it is clear that local adaptation rather than behavioural plasticity is important. My study demonstrates for the first time that sympatry can enhance innate responses to heterospecific alarm cues and how this interacts with phylogenetic relationships to fine-tune anti-predator behaviour.

CHAPTER 7

Discussion

The individual chapters in this thesis present detailed discussions of how the results relate to other studies, therefore in this chapter I will concentrate on how the findings in the separate chapters synthesise to provide a general picture of how *L. stagnalis* modifies its anti-predator behaviour to match its environment. The general aims of this thesis were to ask whether *L. stagnalis* possess innate elements in its anti-predator behaviour, and if innate behaviour could be altered through learning about current predation risk. I investigated whether innate elements of the behaviour showed local modification to predator regime. A selection experiment was then used to find out if population differences were due to genetic adaptation for a constitutive response, and how rapidly this response may evolve under selection. Utilising the selection lines, I then investigated whether there was also a plastic response when snails underwent development under exposure to predator cues. Finally, I addressed the question of whether *L. stagnalis* was able to utilise information from intraguild members to assess predation risk, and whether this response depended on phylogenetic relationships or co-habitation.

The aquatic gastropod *Lymnaea stagnalis* was found to fine-tune its behavioural responses to predator cue paired with alarm cues to match the predator regime it experiences. *Lymnaea stagnalis* was adapted to exhibit an elevated innate response to predation cues from tench when originating from a population naturally encountering these predators (Chapter 3&4: Dalesman et al. 2007b, Dalesman et al. in review). Populations varied in the degree of flexibility to predator presence in the environment following long term exposure to predator kairomones (Chapter 5: Dalesman et al. in prep.). *Lymnaea stagnalis* may also increase perceived risk through cue association learning in response to cues indicative of predator foraging activity (Chapter 2: Dalesman et al. 2006). As well as information on predator presence, *L. stagnalis* is able to use alarm cues from heterospecific gastropods in combination with predator kairomones to assess risk levels, and this perception of risk is higher when species are found in the same water body as the responding population (Chapter 6: Dalesman et al. 2007a).

The degree to which *L. stagnalis* is behaviourally adapted to its local environment is not unexpected as predators may have both a direct impact on fitness through predation, but can also have an indirect effect on fitness through lost opportunities to feed or reproduce (Lima and Dill 1990). The relatively low dispersal abilities of freshwater gastropods, due to the fact that most species are only able to tolerate short periods out of the water due to the risk of desiccation, means that these organisms are unable to escape predation by moving to a less risky environment. Therefore, in order to reduce the chance of predation, *L. stagnalis* needs to adapt to match the local predation risk as closely as possible and to minimise fitness costs.

Lymnaea stagnalis has been found in previous studies to show an induced morphological response to the presence of predator cues (Rundle et al. 2004). However, morphological responses in gastropods are often costly, both in terms of energy diverted from soft body growth and sexual development towards shell excretion (Brookes and Rochette 2007), the costs of carrying a heavier shell, and the potential reduction in space available for soft tissues (Trussell 2000b, Turner 2004, Brookes and Rochette 2007). Therefore using morphological defence as an innate response may be costly if predators are not encountered. Behavioural defences may also have associated costs in terms of maintaining the sensory systems to respond to predators when they are encountered (DeWitt 1998), but relative to morphological defences these costs are likely to be low. Behavioural responses also have very little lag time between induction and display of the response, and are potentially easily reversed, so in a fluctuating environment they can respond rapidly to changes in risk (Gabriel et al. 2005). The rapid changes in perceived risk, both in the rapid increase in anti-predator behaviour in response to predator kairomones, and also a rapid decline in this response, provide an example of the speed with which behavioural changes can respond to the predation environment (Chapter 2: Dalesman et al. 2006).

Innate responses were found to some degree in anti-predator behaviour in all the populations of *L. stagnalis*, however, the responses to tench cues paired with alarm cues were stronger in populations found naturally co-habiting with predatory fish (Chapter 3: Dalesman et al. 2007b). This increased innate response indicates, as expected, that there is selective pressure on animals co-habiting with predatory fish to respond with anti-predator behaviour on their first encounter with these predators, and that these costs outweigh costs of maintaining the sensory and response systems necessary. The innate response toward predation (combined) cues was also present in populations that do not normally encounter predatory fish, though the response to fish cues alone was not. This indicates that either the mechanism to respond to fish cues alone is separate from that used to respond to combined cues, or that there is a threshold in sensory perception that needs to be passed before responses are seen to fish cues alone. Results from a selection experiment, selecting over two generations for high and low crawl out responses in two populations from high (South Drain) and low (Chilton) predator regimes seemed to support the former explanation. The innate response to combined cues was increased to a similar level to that seen in the populations naturally encountering predatory fish, however the response to tench cue alone did not increase in response to this selection in either population (Chapter 4: Dalesman et al. in review). This suggested that the response to tench cue alone is not linked to the response to combined cues, but has been selected for separately. The generally lower level of response to tench cue alone suggests that a non-foraging fish is less of a predation risk than a foraging one.

In this case, selection was used to understand the mechanism for differences between the populations showing different levels of innate behaviour in the F1 generation. F1 behaviour may be affected by transgenerational induction based on the parental environment (Agrawal et al. 1999) or may be due to genetic adaptation as found in the morphological response of a proportion of the population of an intertidal barnacle, *Chthamalus anisopoma*, responding to the presence of a predatory crab (Lively et al. 2000). Manipulation of the response level, by selecting out high and low responding individuals, indicated that there is a strong genetic basis for the degree of crawl-out response seen and that levels of divergence between high and low responding populations can be selected for in only two generations, indicating that rapid microevolution of populations is possible (Chapter 4: Dalesman et al. in review).

This adaptation for a fixed response was altered by exposure to feeding tench, i.e. tench and alarm cues paired together, raising the perceived risk associated with tench cue over short periods. However, exposure to predator cues may not always be paired with alarm cues from conspecifics, or recognised heterospecifics (Chapter 6: Dalesman et al. 2007a). For example, tench may feed on other invertebrate taxa such as insects and crustaceans when gastropods are at low abundance in the environment. Furthermore, tench are dormant and do not feed during winter months, so it is possible that *L. stagnalis* may be exposed to tench cues for considerable periods of time without those cues being paired with recognised alarm cues. To test the potential effect of exposure to tench cue alone, F3 *L. stagnalis* from each of the selection lines were exposed to tench cues from laying onwards (Chapter 5: Dalesman et al. in prep.). The effect of exposure to tench kairomones was to increase the crawl-out response in the low and randomly selected lines, but not in the high selection line. This suggested that either the plastic response to exposure to tench kairomones during development is linked to the mean innate response, such that inheritance of a higher innate response occurs alongside inheritance of a reduced plasticity in the response or, more likely, that there is a 'ceiling effect' occurring (Teuschl et al. 2007), so that the response seen in the high response line is the maximum crawl-out response possible.

The ability to show this type of plastic response to exposure during development means that *L. stagnalis* is not dependent on genetic adaptation of constitutive responses to allow it to respond in a novel environment, but can demonstrate plasticity in response to tench kairomones. The persistence of the plastic response in both low selection lines, and also in a population not naturally exposed to predatory fish (Chilton), indicated that maintaining the ability to show this type of plasticity does not carry high fitness costs, or it is likely it would have been lost from the population (Relyea 2002a, b, Boeing et al. 2006). Potentially there is little cost in maintaining the sensory/recognition systems required to respond to cues, indicating that the presence of predatory fish is a reliable indicator that those fish will be around for at least part of the snail's lifetime. The loss of the innate response in environments where predatory fish are not found, indicates that this does have associated costs, possibly relating to physiological adaptation which is not beneficial when predatory fish are absent.

Cues associated with predator presence also induced responses, even in populations which normally demonstrated a lower innate anti-predator response. This suggests that *L. stagnalis* can maintain flexibility in their anti-predator behaviour following local adaptation. This type of flexibility potentially allows *L. stagnalis* to persist in a novel environment to enable adaptation to occur.

The maintenance of a plastic response to predators not currently co-existing with the population may depend on *L. stagnalis* having overlapped at some point in their evolutionary history with that predator, and is not necessarily the case for species never previously encountered by *L. stagnalis*. The effect of phylogeny on cue evolution may affect the ability to recognise predatory species as it does with recognition of heterospecific alarm cues (Chapter 6: Dalesman et al. 2007a). There is some evidence that responses to predator threat in other gastropods is related to phylogenetic relationships between the predatory species. For example Langerhans and DeWitt (2002) found that *Physella virgata* responded morphologically to several different species of sunfish (*Lepomis cyanellus, L. gibbosus, L. macrochirus, L. megalotis, L. microlophus, Micropterus salmoides*) despite only some of these species being molluscivorous. In this case the response is mal-adaptive as *P. virgata* suffers from reduced growth needlessly; however, in encounters with potential predators such a response may enhance survival. There is also the possibility that the use of cue association to enhance the perceived risk from tench cue may also be used to identify predators that have not previously been encountered. This is common in fish species (Chivers and Smith 1998, Brown 2003, Kelley and Magurran 2003) and has also been found in crustaceans (Hazlett 2003), but has not yet been tested in aquatic gastropods. *Lymnaea stagnalis* would prove a useful model to assess how gastropods may respond to invasive species as both these hypotheses would be easily tested using the methodology developed here.

As suggested so far, the ability of an animal to show induced responses, or to utilise cue association to recognise previously unknown predatory threat, may allow successful colonisation. In freshwater pulmonates there is a high degree of variation in the habitat specialisation between closely related species. Some species are widely distributed between habitats with differing predator regimes (e.g. *L. stagnalis* and *Radix balthica*), whereas other are very specialised, such as *Radix auricularia*, which is only found in large water bodies, and *Omphiscola glabra* that is only found in small temporary water bodies. The inability of some of these species to colonise different habitats, as well as the ability to cope with varying abiotic conditions, may in part be due to slow responses to different predator regimes in each environment. Species comparisons of how responses to cue during development affect behavioural responses to potential predatory species may shed light on why some lymnaeid species are widespread, whereas others are restricted. Comparisons across the phylogeny may be used to shed light on the evolution of mechanisms controlling anti-predator behaviour within this taxonomic group.

So far this work has concentrated on interactions between two species, *L. stagnalis* and tench, however there are many other organisms living in the same environment which have potential to affect or be affected by predatory interactions. Previous studies have shown strong linkages between gastropod prey, their fish predators and macrophyte abundance (Brönmark 1994, Turner 1997), showing that the relationship between predator and prey may affect relationships at other trophic levels. If co-habiting organisms share a common predator then it may be predicted that they will evolve to utilise cue from other intraguild members to assess predation risk in the same way that they use conspecific cues (Snyder 1967). This theory was tested using alarm cues from a number of gastropod species at differing phylogenetic distances from *L. stagnalis*, some found allopatrically and some sympatrically relative to the source population of *L. stagnalis* (Chapter 6: Dalesman et al. 2007a). Alarm cues from phylogenetically close species cause stronger behavioural responses than those of more distantly related species. This relationship between response and genetic distance indicates there may be a gradual mode of evolution in either the signalling chemicals or the way in which they are detected (Jovelin et al. 2003, Symonds and Wertheim 2005, Kelly et al. 2006). However, sympatric species also induced a stronger response than allopatric species, indicating that local adaptation has occurred to enable *L. stagnalis* to respond more strongly to co-habiting species (Chapter 6: Dalesman et al. 2007a).

The data presented here provide a considerable body of evidence for the behavioural adaptation of *L. stagnalis* to local environmental conditions, both in terms of predator regime (Chapter 3: Dalesman et al. 2007b) and also in terms of responses to alarm cues from intraguild members (Chapter 6: Dalesman et al. 2007a). This indicates that *L. stagnalis* may suffer reduced fitness due to a decrease in the accuracy of assessing predation risk associated with encounters with unfamiliar species. This may occur during invasions of predators or intraguild members into its habitat, or when *L. stagnalis* colonises a new habitat. This may be true for other taxa with limited distribution, and may be particularly relevant for freshwater organisms lacking terrestrial dispersal stages.

However, *L. stagnalis* also appears able to respond to changes in its predator environment. Selection on crawl-out behaviour in response to tench cues has the potential to occur rapidly, as has adaptation in response to heterospecific intraguild members. It appears that adaptation to a novel intraguild member has already occurred in the South Drain population of *L. stagnalis* as it responds more strongly to alarm cues from the invasive snail, *Physella acuta*, now found living sympatrically with this population, than to a closely related native species, *Physa fontinalis*, which is allopatric. Other evidence for local adaptation having occurred within the South Drain population is that they show a low response to *Lymnaea fusca*, a closely related allopatric species, despite this species living sympatrically with another *L. stagnalis* population (Chilton Moor) less than 1km away.

When maintained at 20°C an unexpected result from the selection experiment was the lack of a difference in the F1 behavioural responses between the South Drain (high risk) and Chilton (low risk) populations as might have been predicted from the differences between these populations found at 15°C (Chapter 3: Dalesman et al. 2007b). Analysis of the crawl out response comparing the two populations at 15°C and 20°C show that the crawl out response decreased with an increase in temperature in the South Drain population, and increased in the Chilton population (Appendix 3). It is impossible to tell, based on the data presented, whether the difference in crawl out response in each population between 15°C and 20°C is a response to development under different temperature regimes, or a response to the temperature purely during the behavioural trials. However it is clear that temperature can, potentially, have a significant effect on the way in which *L. stagnalis* responds to potential predation threats.

One way in which temperature may affect crawl out behaviour is that dissolved oxygen decreased significantly between 51.4% at 15°C and 48.9% at 20°C ($t = 2.84$, $P = 0.010$, d.f. = 21). As *L. stagnalis* is an air breathing pulmonate it may spend more time at the waters surface in response to hypoxic conditions, where dissolved oxygen makes respiration via the membrane less affective. The metabolic rate is also likely to increase at higher temperatures, increasing respiratory rates (Sidorov 2005), which would then increase oxygen demand and surfacing behaviour, and may explain the increase in crawl-out behaviour in the Chilton population. A previous study by Sidorov (2003) on the effects of temperature on respiration and defensive behaviour found that above 14-16°C despite increases in respiration and locomotion, defensive behaviour was inhibited. This reduction in defensive behaviour was suggested to be due to temperature-dependent reactions in the neurons underlying the behaviour. This is potentially the reason for the reduction in defensive crawl out behaviour seen in the South Drain population at the higher temperature.

Lymnaea stagnalis populations may have evolved divergent physiological responses to increased temperature resulting in different behavioural responses. Physiological stress has been demonstrated to alter anti-predator behaviour significantly, either altering the level of anti-predator behaviour (Nicieza 2000, Sidorov 2003), or altering the type of behaviour seen (Villagra et al. 2002). What is clear from these results, and also those of Rundle *et al.* (2004) is that abiotic factors can have a strong influence on anti-predator traits in *L. stagnalis*. The reduction in crawl-out behaviour demonstrated by the South Drain population suggests that an increase in summer temperatures may affect the ability of this species to demonstrate anti-predator behaviours, and hence may impact on the population.

Aquatic gastropods provide an ideal taxonomic group in which to investigate how the mechanisms used to control behavioural and morphological defences may affect the way in which an animal can adapt to differing environments, both in terms of interactions with novel species and their ability to cope with a changing abiotic environment. The interspecific variation in their colonisation abilities and a relatively well defined phylogeny means that cross-species comparisons can be used to investigate the evolution of various traits. The work presented in this thesis provides a number of novel ways to utilise a simple experimental design to investigate the genetic adaptation and plasticity of anti-predator behaviour in pulmonates, which could easily be extended to provide cross species comparisons.

Conclusions

The work presented here demonstrates that *Lymnaea stagnalis* has evolved to fine-tune its behavioural responses to the predation environment it experiences. Individuals exhibited genetic adaptation for fixed responses on first encounter with a potential predator, which relates to the predator regime experienced in their natural populations (Chapter 3: Dalesman et al. 2007b). This adaptation has the potential to occur within just two generations which may allow rapid micro-evolution within populations on encountering novel conditions (Chapter 4: Dalesman et al. in review). *Lymnaea stagnalis* also demonstrated a more rapid induced response to the presence of predators, both through exposure to fish cues during development (Dalesman et al. in prep.), and in increased perception of risk relating to cues associated with predator foraging activity (Chapter 2: Dalesman et al. 2006). These induced responses may allow them time to adapt on entering an environment with an alternate predator regime, and also potentially allow recognition of predators not previously encountered in their evolutionary history. As well as the ability to utilise predator cues, *L. stagnalis* can also use alarm cues from heterospecifics to identify predation risk from species with a common predator (Chapter 6: Dalesman et al. 2007a). This ability appears to have also undergone local adaptation, such that responses to sympatric species are stronger than those to allopatric species, though the mechanism controlling this needs further investigation.

L. stagnalis demonstrates many of the features shown in other aquatic species of local adaptation and induced behavioural responses from cue exposure. These results apply particularly where organisms are restricted in their distribution to isolated water bodies, and therefore cannot escape predation by dispersal, but have to find other means of reducing predation pressure through changes in behaviour, morphology and life history. *Lymnaea stagnalis* has proven to be a good model to investigate the mechanisms behind these adaptations to life with restricted distribution, and provides insight into how well such organisms may cope with change in their environment. The relative ease with which pulmonate snails can be obtained and reared in the laboratory suggests they will provide good model species to investigate aspects of local genetic adaptation and plasticity further.

Appendices

Appendix 1

Strength of tench cue used.

The production of tench odour was tested to assess how long tench needed to be left in a tank to produce sufficient odour to result in *Lymnaea stagnalis* producing an anti-predator response. Three tench of approximately 10cm long were placed in 4 litres of artificial pond water for either 5 minutes, one hour or 24 hours. Juvenile *L. stagnalis* from Matford Park, 6±0.5mm shell height, were acclimated to the behavioural chambers in 630ml of artificial pond water for 1 hour prior to behavioural trials. Thirty-six snails were tested on each of 3 days, with 9 snails randomly assigned to one of four treatments (giving a total of 27 snails per treatment). 70ml was added to each chamber (to give 10% odour addition), either from 5 minute cue, 1 hour cue, 24 hour cue or control (artificial pond water with no tench). Position of individual snails was then noted every 5 minutes for 3 hours. Crawl out behaviour was used to analyse the anti-predator behaviour of *L. stagnalis* as it has been found to be their primary anti-predator response to tench cue.

No significant difference was found between the 3 odour treatments in either latency to crawl out, the proportion of time spent crawled out or the number of individuals that crawled out. Odour production of 1 hour was chosen as the most convenient time period for all future experiments.

Appendix 2

Table A2.1: Results of pair-wise comparisons using ANOSIM between field sites (see Chapter 1). R values for each pair-wise comparison are below the diagonal and P values above the diagonal (bold = significant difference).

	South Drain	**Sowy River**	**N. Moor Main Drain**	**Chilton**	**Little Hook**	**Wistaria**	**N. Moor Small drain**
South Drain		**0.029**	**0.029**	**0.029**	**0.029**	**0.029**	**0.029**
Sowy River	0.51		**0.029**	0.143	**0.029**	0.286	0.286
N. Moor Main Drain	0.865	0.438		0.057	**0.029**	0.400	**0.029**
Chilton	0.49	0.219	0.344		0.457	0.143	0.657
Little Hook	0.781	0.563	0.563	0.031		**0.029**	**0.029**
Wistaria	0.76	0.083	0.021	0.156	0.323		0.057
N. Moor small	0.833	0.167	0.685	-0.148	0.519	0.389	

Table A2.2: Pearson correlation value (upper number in each cell) and associated P-value (lower number in each cell) between environmental variables measured at six sites on the Somerset Levels. Bold font = significant correlation between variables.

Variable	$[Mg^{2+}]$ (mg/l)	$[Ca^{2+}]$ (mg/l)	Nitrate (mg/l)	Phosphate (mg/l)	Width (m)	Depth (cm)	pH	%O_2	O_2 mg/l	µS adj. for temperature	Temperature (°C)
$[Ca^{2+}]$ (mg/l)	**0.690** **<0.001**										
Nitrate (mg/l)	-0.141 0.484	-0.052 0.796									
Phosphate (mg/l)	**-0.417** **0.030**	-0.238 0.232	0.268 0.177								
Width (m)	-0.222 0.266	0.003 0.990	-0.212 0.288	0.145 0.469							
Depth (cm)	-0.201 0.316	-0.004 0.985	-0.214 0.283	0.161 0.422	**0.896** **<0.001**						
pH	-0.050 0.806	0.110 0.585	-0.308 0.118	-0.113 0.575	**0.678** **<0.001**	**0.662** **<0.001**					
%O_2	0.319 0.105	**0.470** **0.013**	-0.106 0.600	-0.347 0.076	**0.456** **<0.001**	**0.504** **0.007**	**0.635** **<0.001**				
O_2 mg/l	0.374 0.054	**0.543** **0.003**	-0.095 0.639	-0.368 0.059	**-0.235** **<0.001**	**0.463** **0.015**	**0.578** **0.002**	**0.984** **<0.001**			
uS adj. for temperature	**0.712** **<0.001**	**0.660** **<0.001**	-0.048 0.811	-0.170 0.396	-0.235 0.239	-0.222 0.266	-0.214 0.283	0.103 0.608	0.168 0.402		
Temp.(°C)	**-0.462** **0.015**	**-0.612** **0.001**	-0.052 0.798	0.334 0.088	0.171 0.394	0.163 0.418	0.178 0.374	-0.296 0.134	**-0.441** **0.021**	**-0.421** **0.029**	
Salinity p.p.t.	**0.618** **0.001**	**0.594** **0.001**	-0.111 0.582	-0.135 0.503	-0.214 0.285	-0.202 0.314	-0.295 0.135	0.066 0.743	0.130 0.517	**0.952** **<0.001**	**-0.447** **0.019**

Table A2.3: BIOENV results, correlation value for individual environmental variables with assemblage.

Environmental variable	Correlation
[Mg^{2+}] (mg/l)	0.304
pH	0.275
Temperature (°C)	0.267
Width (m)	0.184
Depth (cm)	0.165
[Ca^{2+}] (mg/l)	0.143
Salinity p.p.t.	0.135
%O_2	0.063
μS (adj. for temp.)	0.050
O_2 (mg/l)	0.031
Phosphate (mg/l)	-0.094
Nitrate (mg/l)	-0.192

Appendix 3

The effects of temperature on crawl out behaviour.

It was noted during the F1 behavioural trials at 20°C the differences seen in the degree of crawl out behaviour did not differ between the high risk (South Drain) and low risk (Chilton) populations. This result appeared to contradict the previous findings that the innate behaviour of F1 *L. stagnalis* differs depending on the predator regime experienced by the population of origin (Chapter 3: Dalesman et al. 2007b). To test the responses from the F1 behavioural trials at 20°C were compared with the results from behavioural trials of F1 South Drain and Chilton populations done for population comparison at 15°C. A four-way ANOVA carried out in GMAV on both time spent crawled out and latency to crawl out was used analyse differences between the two temperatures, with post-hoc Student-Newman-Keul (SNK) tests used to assess the direction of these differences.

The effect of rearing snails in 20°C as opposed to 15°C significantly altered the F1 crawl out behaviour, and the way in which the behaviour was changed differed depending on the source population (Chapters 3&4: Dalesman et al. 2007b, in review). Overall the time spent crawled out in response to combined cues was significantly greater than to either cue presented alone, and was significantly greater in the population originating from South Drain compared to the population originating from Chilton, as predicted from the predator regime each population experiences (Table 3.1; ANOVA: population*A*T: $F_{1,368} = 6.14$, $P = 0.014$; SNK: $P<0.05$). However the effect of temperature on the response the tench cue differed between the populations; the time spent crawled out decreased in the South Drain population in 20°C compared to 15°C, whereas it increased in the Chilton population following development at 20°C (Table A3.1; ANOVA: temperature*population*T: $F_{1,368} = 15.91$, $P<0.001$; SNK: $P<0.05$). The latency to crawl out was shortest in response to combined cues, but did not differ significantly between the populations (Table A3.1; ANOVA: A*T: $F_{1,368} = 93.58$, $P<0.001$). However, at 15°C the latency to crawl out in response to tench cue was significantly shorter in the South Drain population, as predicted by the predator regime experienced, but at 20°C there was no difference in latency between the South Drain and Chilton populations in their response to tench cue (Table A3.1; ANOVA: temperature*population*T: $F_{1,368} = 7.58$, $P = 0.006$).

Table A3.1: Results from 4-way ANOVA on time spent crawled out of the water and latency to crawl out. Factors used are: temperature (15°C/20°C), population (South Drain/Chilton), alarm cue (present/absent) and tench cue (present/absent). N = 24 for all treatment combinations.

Source	**d.f.**	**Proportion of time**			***Latency***		
		Mean square	**F**	**P**	**Mean square**	**F**	***P***
Temperature (Temp)	1	0.062	0.85	0.357	0.208	1.97	*0.162*
Population (Pop)	1	1.105	15.14	<0.001	0.945	8.93	*0.003*
Alarm cue (A)	1	10.299	141.13	<0.001	9.647	91.10	*<0.001*
Tench cue (T)	1	17.238	236.21	<0.001	19.955	188.42	*<0.001*
Temp*Pop	1	1.493	20.46	<0.001	1.799	16.98	*<0.001*
Temp*A	1	0.018	0.24	0.622	0.028	0.26	*0.609*
Temp*T	1	0.082	1.12	0291	0.157	1.48	*0.225*
Pop*A	1	0.291	3.98	0.047	0.081	0.77	*0.382*
Pop*T	1	1.197	16.40	<0.001	0.812	7.67	*0.006*
A*T	1	9.851	134.99	<0.001	9.910	93.58	*<0.001*
Temp*Pop*A	1	0.175	2.40	0.122	0.102	0.96	*0.327*
Temp*Pop*T	1	1.161	15.91	<0.001	0.802	7.58	*0.006*
Temp*A*T	1	0.002	0.03	0.868	0.007	0.06	*0.805*
Pop*A*T	1	0.448	6.14	0.014	0.313	2.96	*0.086*
Temp*Pop*A*T	1	0.152	2.08	0.150	0.022	0.20	*0.653*
Total	*383*						

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Cue association and antipredator behaviour in a pulmonate snail, *Lymnaea stagnalis*

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Associative learning may help to offset costs of unnecessary escape behaviour by providing accurate information about the current risk to potential prey. We investigated innate antipredator behaviour and cue association learning in naïve gastropods. Juvenile laboratory-reared great pond snails, *Lymnaea stagnalis* (L.), were exposed to odour cues from a natural predator, tench, *Tinca tinca* (L.), and crushed conspecifics. The snails showed an innate antipredator behaviour to odour from *T. tinca*, by crawling above the water line (crawl-out response). This crawl-out response was significantly increased in the presence of alarm cues (crushed conspecifics). In a second experiment, juvenile *L. stagnalis* were exposed to tench odour and alarm cues in aquaria before being tested in behavioural assays. The behavioural responses to tench cue alone were similar to those seen in response to tench plus alarm cues presented together during the first experiment. Hence, *L. stagnalis* is apparently capable of relating potential predation risk to recent experience. In a final experiment snails were removed from pre-exposure cues for periods of 1, 4 and 8 days prior to behavioural assays. A raised level of avoidance persisted for at least 8 days, suggesting that this behaviour may be retained over timescales relevant to predation risk in the natural environment. The ability of organisms to modify antipredator behaviour based on recent experience, as found in *L. stagnalis*, would allow costs associated with unnecessary responses to be reduced while still allowing the organisms to avoid active predators.

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Failure to respond to a potential predator may be fatal; however, unnecessary escape behaviour may have direct energetic costs as well as costs associated with reduced opportunity to feed or reproduce (Lima & Dill 1990). Sensory information obtained about a predator may assist an organism in assessing the potential risk accurately and therefore reduce these costs (Lima & Dill 1990; Chivers & Smith 1998). A range of sensory inputs can be used in predator detection; however, where other cues are impaired by environmental or physiological constraints, for instance visual cues in an aquatic system, chemical cues may provide an alternative (Dodson et al. 1994). Predator-released kairomones (Dicke & Sabelis 1988) can give information about the presence, proximity, physiological state and diet of potential predators (Kats & Dill 1998). Injury-released chemical cues from conspecifics frequently induce an antipredator response in aquatic taxa (Wisenden 2000). However, although a good indication of potential threat in isolation, these types of cue may not provide accurate information about the nature of current risks, and so may elicit inappropriate antipredator responses.

Several studies have shown that combining alarm cues produced by injured conspecifics with predator kairomones elicits the greatest antipredator response in organisms, suggesting that this represents the greatest perceived risk. In experiments, this combination of cues either takes the form of the predator feeding on conspecifics (Crowl & Covich 1990; Loose & Dawidowicz 1994; Sih & McCarthy 2002; Turner & Montgomery 2003; Jacobsen & Stabell 2004) or predator cues paired with crushed conspecific, thought to simulate a predation event (Turner 1997; DeWitt et al. 1999; Rundle & Brönmark 2001; Cotton et al. 2004). Some prey organisms that respond to predator cues paired with conspecific cues do not respond to predator cues alone (Turner 1996; Jacobsen & Stabell 2004). An important question is whether this is due to an inability to

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recognize the predator or to a lower perceived risk from a nonfeeding predator. The ability of gastropods to differentiate between the types of predator present from cues paired with injured conspecifics suggests that the latter may be the case in some predator–prey systems. For instance, the freshwater snails *Physella gyrina* and *P. integra* are able to differentiate between cues from different predators, crayfish, *Orconectus rusticus*, and pumpkinseed fish, *Lepomis gibbosus*, when presented with crushed conspecific snail, and respond with appropriate antipredator behaviour (Turner et al. 1999; Bernot & Turner 2001).

Experience of predation cues is an important element in the development of antipredator behaviour in a wide range of vertebrates as well as invertebrates. Learning from experience of predation is integral to most fish species' ability to recognize predators (Brown 2003; Kelley & Magurran 2003). For instance, fathead minnows, *Pimephales promales*, learn to recognize a potential predator, northern pike, *Esox lucius*, from chemical cues produced by pike feeding on minnows (Mirza & Chivers 2001). Learning about predators through diet information, or pairing of predator cues with damaged conspecifics, has also been noted in several invertebrates including damselflies, *Enallagma* spp. (Chivers et al. 1996), crayfish (Hazlett et al. 2002), whelks, *Buccinum undatum* (Rochette et al. 1998) and an aquatic flatworm, *Dugesia dorotocephala* (Wisenden & Millard 2001). In these cases, it was unclear whether the prey species were learning to identify a potential predator per se, or whether the association between cues was providing information about the perceived risk from the predator.

The giant pond snail, *Lymnaea stagnalis* (L.), is slow moving relative to many molluscivorous predators, and lives in an aquatic environment where visual information may often be hindered by turbidity or dense vegetation. It responds to chemical cues from potential predation risk by either sheltering in crevices or crawling above the water line (Snyder 1967; Rundle & Brönmark 2001). As such *L. stagnalis* is predicted to use chemical cues as an important source of information about predators (Wisenden 2000), which makes it an ideal model organism to study the way in which chemical cues can be used to assess predation risk. Our aims in this study were to use naïve laboratory-reared snails to (1) determine the extent of innate responses to predator cues, (2) assess the ability of *L. stagnalis* to form cue associations, (3) determine the effect of these associations on behavioural responses, and (4) determine the longevity of these associations. We predicted that (1) *L. stagnalis* would respond to higher risk by displaying a stronger antipredator response, (2) the perceived risk from predator cues would be enhanced by cue association with a simulated predation event, and (3) perceived risk would diminish over time from exposure to a simulated predation event.

METHODS

Study Organisms

Adult *L. stagnalis* were collected from Matford Park canal, U.K. (5°42′N, 03°31′W) in April 2004. In the laboratory, adults were kept in 6-litre Savic plastic aquaria (Aquatics online, Bridgend, U.K.) in 4 litres of aerated artificial pond water (ASTM 1980) with 90 mg/litre [Ca^{2+}] (Rundle et al. 2004). They were maintained at 15 ± 1°C under ambient light levels, and fed on Iceberg lettuce. Experimental snails were bred from egg masses laid at least 2 months after collection, to minimize the potential influence of adult experience on juvenile development and behaviour. Juvenile snails were then maintained separately in the same conditions as the adults. Individuals with a standard spire height of 6 ± 0.5 mm were used for behavioural experiments.

Tench, *Tinca tinca*, were taken from a laboratory stock originally obtained from Emperor Tropicals & Water Garden Centre, Plymouth, U.K. They were maintained in aerated and filtered water at 15 ± 1°C under ambient light levels in 25-litre tanks at a stocking density of 12 fish per tank (0.006 kg/litre). Gravel substrate and shelters positioned in the tank were used to mimic natural conditions. The tench were fed Nutri-flake (Hozelock Cyprio, Aylesbury, U.K.), which contains no mollusc extracts, and hence avoided potential dietary cues affecting the trials. No experimental manipulations were carried out on these fish and the welfare of the fish was carefully considered throughout. After the trials, the tench were retained as laboratory stock.

Odour Production

Predator and alarm cues were produced in the same way in all the experiments. We used *T. tinca* to produce the predator cue because they are a known natural predator of freshwater gastropods (Brönmark 1994). We produced predator cue water by placing three *T. tinca* (length 10 ± 1 cm) into 4 litres of aerated artificial pond water for 1 h, alarm cue by mixing three crushed juvenile *L. stagnalis* (6 ± 0.5 mm) into 4 litres of aerated artificial pond water, and predator plus alarm cue by mixing three crushed *L. stagnalis* (6 ± 0.5 mm) into 4 litres of tench cue water. We killed the snails for the alarm cue by instant crushing in a pestle and mortar, as physical methods of killing are thought to be the least distressing for the animal (Close et al. 1996). Owing to the small size of the snails, the ease with which their shells can be crushed and the potential alteration of chemical cues with other methods, we considered this the most appropriate way to kill the snails.

Behavioural Assay

We carried out behavioural trials in the same way for all experiments under laboratory conditions analogous to the conditions in which the snails were maintained. Behavioural chambers consisted of a white plastic dish (165 mm in diameter × 60 mm deep; A.W. Gregory & Co. Ltd., London, U.K.), with a longitudinally sectioned white PVC pipe (36 mm long, 30 mm in diameter) attached open side down to the centre with nontoxic sealant to provide a refuge. Juvenile snails were acclimatized to behavioural chambers for 24 h in 630 ml of artificial pond water prior to behavioural assays. We added 70 ml of cue water to each chamber at the start of the behavioural assay to give a 10% final concentration of cue water. Preliminary trials with predator cue alone showed that this was an adequate odour strength to initiate an antipredator response in *L. stagnalis*.

We recorded the position of each snail within the behavioural chambers every 5 min for 2 h after adding the cue. Crawl-out behaviour (where the snail moved above the water line) was the main antipredator response of *L. stagnalis* to the predator, with the refuge not used; hence only results from crawl-out behaviour are presented here. To analyse crawl-out behaviour we used two variables: the proportion of total time spent crawled out of the water; and the latency to crawl-out, calculated as the proportion of total time during the trial before crawl-out behaviour was observed (Cotton et al. 2004). Proportion of total time spent crawled out was used in conjunction with the time at crawl-out because snails occasionally returned beneath the water line. The results of a power analysis on our preliminary study indicate that using 20 or more individuals per treatment gives an 80% or higher probability of detecting an effect of treatment.

Risk Level and Avoidance Behaviour

After acclimatization of the snails to the behavioural chambers, we used four treatments to assess the response of *L. stagnalis* to varying degrees of predation risk: (1) control (untreated artificial pond water); (2) alarm cues alone (crushed conspecifics); (3) predator odour alone; and (4) predator odour plus alarm cues. We predicted that the control would represent the lowest risk level and predator plus alarm cues the highest, with predator or alarm cues presented individually being perceived as an intermediate risk. We randomly assigned individuals to a treatment level such that equal numbers of snails were exposed to each treatment per trial ($N = 24$ snails per treatment combination).

Cue Association

To assess whether a simulated predation event (predator and alarm cues together) increased the perceived risk from a predator, we exposed snails to a conditioning treatment where both cues were presented together, followed by exposure to predator cues alone during the behavioural assay. This would represent experience of a predation event in the environment followed by the predator returning to the environment without subsequent predation occurring.

Juvenile snails were pre-exposed for 48 h, maintained in 1 litre of water in 1.6-litre Savic tanks in one of four conditioning treatments: (1) control (untreated artificial pond water); (2) alarm cues alone (crushed conspecifics); (3) predator odour alone; and (4) predator odour plus alarm cues. Cue water was produced as in the behavioural assays and again added at a concentration of 10% by volume.

After pre-exposure, individual snails were randomly allocated and acclimatized to behavioural arenas in control water for 24 h. Before the behavioural assays, we added either control water or predator cue to the behavioural arena, so that for each trial half of the snails that had been conditioned to each treatment were exposed to the control or predator cue. We used 192 snails; 48 were exposed to each conditioning treatment, and then half of each group was exposed to predator odour during behavioural assays and half to a control, resulting in 24 snails for each treatment combination.

Persistence of Cue Association

To assess the persistence of cue association, we pre-exposed juvenile snails as before for 48 h to either control water (artificial pond water only) or predator odour plus alarm cues. They were then either acclimatized in behavioural arenas for 24 h, placed into control water for 3 days and then acclimatized for 24 h, or placed into control water for 7 days then acclimatized for 24 h. This resulted in total periods of 1, 4 and 8 days since cue exposure before the behavioural assay. Snails were randomly allocated to a behavioural arena, and exposed to either control water or predator odour during the behavioural assay ($N = 23$ snails per treatment combination).

Statistical Analysis

We analysed data as balanced ANOVAs using GMAV5 (Institute of Marine Ecology, University of Sydney, Sydney, Australia) except in the case of the persistence of cue association experiment which was initially analysed with a general linear model procedure in MINITAB13 (Minitab Ltd., Coventry, U.K.) owing to an unbalanced design between data collection trials. As no significant effect of trial was found, we removed trial as a factor and carried out further analysis using a balanced ANOVA in GMAV5. All proportion data were square-root arcsine transformed before analysis (Underwood 1997). Student–Newman–Keuls tests (SNK) were used for post hoc comparisons.

Log-likelihood tests (*G* tests) were used to assess the number of snails crawling out in response to different treatments; data were $N + 1$ transformed when zeros were present, and adjusted by Williams' correction (Sokal & Rohlf 1995). α was corrected using the Bonferroni correction to $\alpha' < 0.025$ to test cue association, and $\alpha' < 0.0125$ to test the persistence of cue association, as multiple comparisons were required to analyse the data (Sokal & Rohlf 1995).

RESULTS

Risk Level and Avoidance Behaviour

The number of *L. stagnalis* crawling out differed significantly between treatment levels (*G* test: $G_3 = 66.750$, $P < 0.001$): no snails crawled out in either the control or alarm cue treatments; however, 15 snails crawled out in response to the predator cue alone, and 21 snails crawled out in response to predator plus alarm cues.

Latency to crawl-out was significantly shorter in response to predator plus alarm cues compared to the

Table 1. Latency to crawl-out and proportion of total time spent crawled out in response to exposure to tench cues and conspecific alarm cues

		Latency			Proportion of time		
Source	*df*	Mean square	*F*	*P*	Mean square	*F*	*P*
Trial (Tr)	2	565.79	1.53	0.225	701.01	2.49	0.089
Alarm cue (Al)	1	2600.47	6.99	0.010	6511.94	23.17	<0.001
Tench cue (Te)	1	52 921.62	142.16	<0.001	31 123.61	110.74	<0.001
Tr*Al	2	647.53	1.74	0.182	127.22	0.45	0.638
Tr*Te	2	565.79	1.52	0.225	701.01	2.49	0.089
Al*Te	1	2600.47	6.99	0.010	6511.94	23.17	<0.001
Tr*Al*Te	2	647.53	1.74	0.182	127.22	0.45	0.638
Total	95						

$N = 24$ for all treatment combinations.

predator cue alone, indicating a more rapid response with increased risk (SNK test: $P < 0.05$; Table 1, Fig. 1a). The proportion of time spent crawled out of the water was significantly greater in response to the predator cue paired with alarm cue than to the predator cue alone (SNK test: $P < 0.05$; Table 1, Fig. 1b).

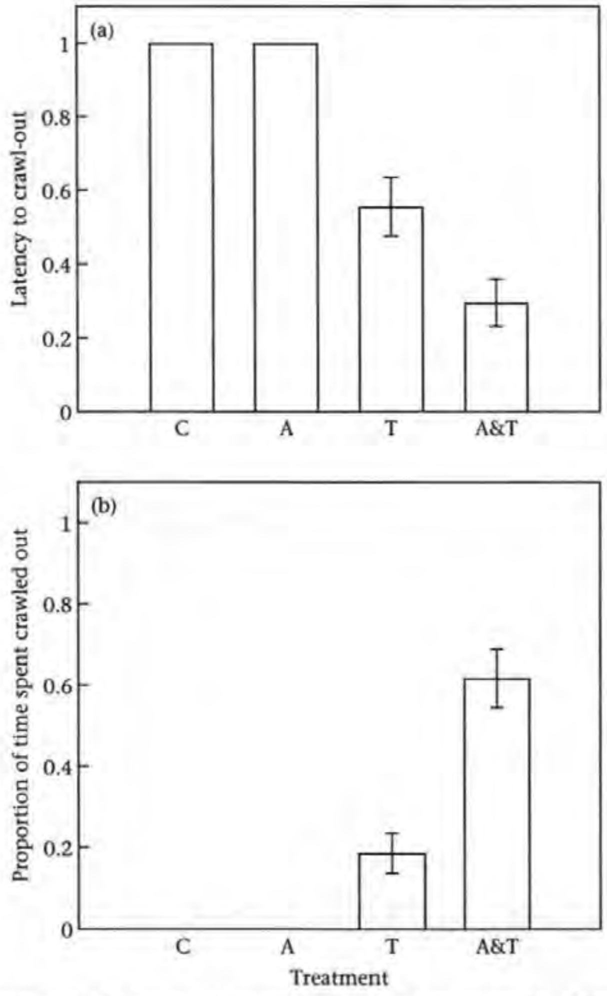

Figure 1. The mean ± SE (a) latency to crawl-out as a proportion of the 2-h assay and (b) proportion of time spent out of the water by *L. stagnalis* in response to different treatments: control (C), conspecific alarm cues alone (A), predator odour alone (T) and alarm plus predator cues (A&T).

Cue Association

Cue association significantly affected the response of *L. stagnalis* to predator cues (Fig. 2). SNK tests ($P < 0.05$) showed that the effect of preconditioning to predator

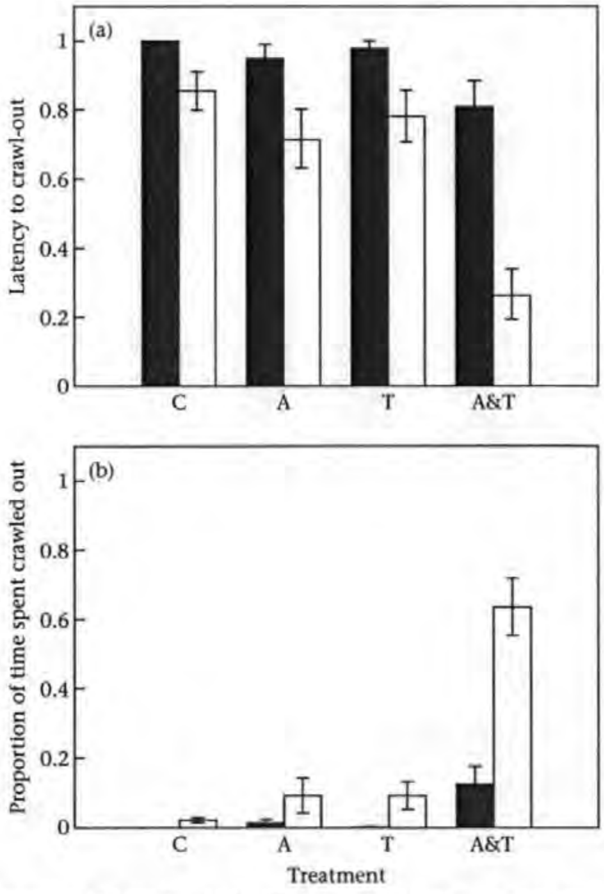

Figure 2. The mean ± SE (a) latency to crawl-out as a proportion of the 2-h assay and (b) proportion of time spent out of the water by *L. stagnalis* responding to control (■) and predator cues (□) after exposure to: control (C), conspecific alarm cues alone (A), predator odour alone (T) and alarm plus predator cues (A&T).

plus alarm cue significantly decreased the latency to crawl-out compared to the control or either cue presented alone (Table 2, Fig. 2a). There was a significant effect of trial on latency to crawl-out (Table 2). However, this was significant only in two pairwise tests between trials, so is not considered to have an overall impact on the results. The proportion of time spent crawled out was significantly greater in response to the predator cue during behavioural assays when *L. stagnalis* had been preconditioned to the predator paired with alarm cue than when individuals had been preconditioned to the control or either cue alone (SNK test: $P < 0.05$; Table 2, Fig. 2b). There was a significant effect of trial, although this was caused by a change in the magnitude of response on only one of the eight trials on individuals that had been preconditioned to alarm cues, so is not considered to have an overall effect on the results (SNK test: $P < 0.05$; Table 2).

Overall, significantly more snails crawled out when exposed to the predator cue during behavioural assays than when exposed to the control during behavioural assays (G test: $G_1 = 30.974$, $P < 0.001$). The number of snails crawling out in response to both the control and predator cue during behavioural assays was significantly greater when they had been pre-exposed to the alarm plus predator cue compared to the control or either cue presented alone (G test: control: $G_3 = 10.307$, $P = 0.016$: predator: $G_3 = 30.324$, $P < 0.001$).

Persistence of Cue Association

Latency was always decreased when predator cues were present during the behavioural assay compared to the control, regardless of the conditioning treatment (SNK test: $P < 0.05$; Table 3, Fig. 3a). However, pre-exposure to predator plus alarm cues significantly decreased the latency to crawl-out in response to the predator cue during behavioural assays up to, and including the 8 days duration compared to individuals pre-exposed to the control (SNK test: $P < 0.05$; Table 3, Fig. 3a). Pre-exposure treatment had no significant effect when snails were exposed to the control during the behavioural assay, and duration since pre-exposure to cues did not significantly affect the latency to crawl-out (Table 3).

The proportion of time spent crawled out was significantly greater in response to the predator cue when *L. stagnalis* had been exposed to predator plus alarm cues compared to any of the other treatments (SNK test: $P < 0.05$; Table 3, Fig. 3b). This significant increase in crawl-out response to predator cues persisted for 8 days after exposure to alarm and predator cues compared to the other treatments. However, the magnitude of the response decreased with duration from pre-exposure, with the proportion of time spent crawled out being significantly lower 8 days after pre-exposure compared to only 24 h after pre-exposure (SNK test: $P < 0.05$; Table 3). The number of individuals showing crawl-out behaviour in both control and predator groups showed no significant decline between 24 h and 8 days (control followed by control; G test: $G_2 = 5.565$, $P = 0.062$; control followed by predator cue: $G_2 = 0.208$, $P = 0.901$; alarm plus predator followed by control: $G_2 = 1.013$, $P = 0.602$: alarm plus predator followed by predator: $G_2 = 2.672$, $P = 0.263$).

DISCUSSION

Risk Level and Avoidance Behaviour

The antipredator response of naïve laboratory-reared F1 *L. stagnalis* to the predator cue alone shows that this snail species has an innate ability to recognize *T. tinca* kairomones and responds with antipredator behaviour. Innate responses to predator threat can be the result of genetic adaptation to recognize potential predators (Åbjörnsson et al. 2004) or a transgenerational influence on offspring

Table 2. Latency to crawl-out and proportion of total time spent crawled out in response to exposure to tench cues after exposure to tench and/or conspecific alarm cues 24 h prior to behavioural assays

		Latency			Proportion of time		
Source	*df*	Mean square	*F*	*P*	Mean square	*F*	*P*
Trial (Tr)	7	1594.82	2.91	0.007	817.16	2.90	0.008
Pre-exposure to alarm cue (PrA)	1	14437.83	26.37	<0.001	12500.61	44.44	<0.001
Pre-exposure to tench cue (PrT)	1	8885.48	16.23	<0.001	11009.90	39.14	<0.001
Tench cue during behavioural assay (Te)	1	25262.17	46.15	<0.001	13111.73	46.61	<0.001
Tr*PrA	7	1048.81	1.92	0.072	774.09	2.75	0.011
Tr*PrT	7	441.83	0.81	0.583	263.74	0.94	0.480
Tr*Te	7	447.94	0.82	0.574	298.71	1.06	0.392
PrA*PrT	1	5206.34	9.51	0.003	7548.35	26.83	<0.001
PrA*Te	1	3148.68	5.75	0.018	4556.87	16.20	<0.001
PrT*Te	1	2242.74	4.10	0.045	4800.66	17.06	<0.001
Tr*PrA*PrT	7	245.29	0.45	0.870	143.28	0.51	0.826
Tr*PrA*Te	7	504.09	0.92	0.493	327.93	1.17	0.327
Tr*PrT*Te	7	306.20	0.56	0.788	218.58	0.78	0.608
PrA*PrT*Te	1	1487.72	2.72	0.102	2985.55	10.61	0.001
Tr*PrA*PrT*Te	7	237.55	0.43	0.879	170.45	0.61	0.750
Total	191						

$N = 24$ for all treatment combinations.

Table 3. Latency to crawl-out and proportion of time spent crawled out in response to exposure to tench cues after pre-exposure to tench plus conspecific alarm cues at varying durations, 24 h, 4 days and 8 days prior to behavioural assay

		Latency			Proportion of time		
Source	*df*	Mean square	*F*	*P*	Mean square	*F*	*P*
Duration (Du)	2	1691.19	2.97	0.053	1403.60	4.07	0.018
Pre-exposure to alarm plus tench cue (Pr)	1	12 189.19	21.40	<0.001	7067.22	20.51	<0.001
Tench cue during behavioural assay (Te)	1	57 709.32	101.33	<0.001	25 736.90	74.71	<0.001
Du*Pr	2	247.89	0.44	0.648	950.91	2.76	0.065
Du*Te	2	653.22	1.15	0.319	1353.53	3.93	0.021
Pr*Te	1	8583.39	15.07	<0.001	6589.99	19.13	<0.001
Du*Pr*Te	2	225.54	0.40	0.674	1452.64	4.22	0.016
Total	275						

Trial is excluded from the model as no significant effect was found using a general linear model. $N = 23$ for all treatment combinations.

(Agrawal et al. 1999). The long time for which adults were held in the laboratory prior to production of F1 juveniles for experimentation in our study potentially reduced the effect of maternal experience on offspring behaviour. However, transgenerational effects of maternal exposure to a dipteran phantom midge predator, *Chaoborus flavicans*, on offspring morphology have been found to persist in the F2 generation of *Daphnia cucullata* (Agrawal et al. 1999).

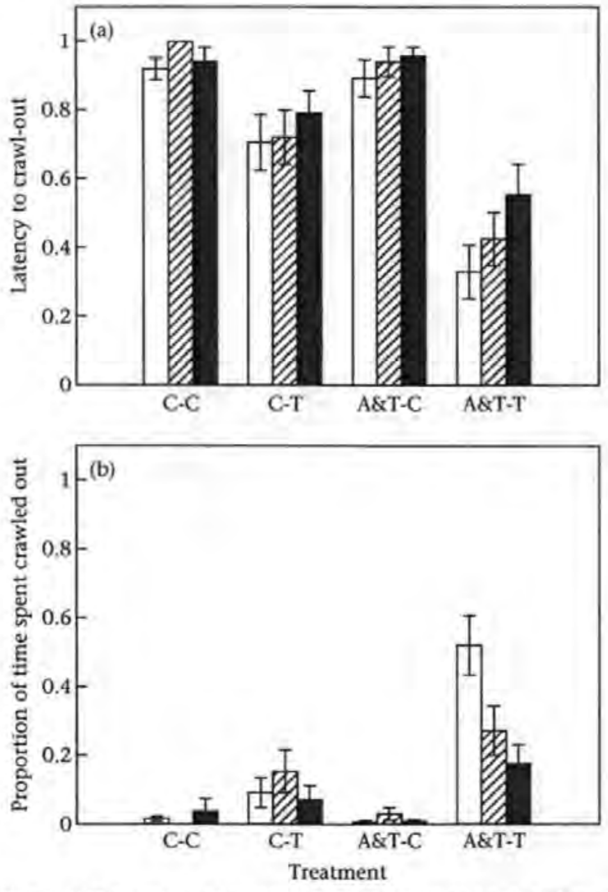

Figure 3. The mean ± SE (a) latency to crawl-out as a proportion of the 2-h assay and (b) proportion of time spent crawled out by *L. stagnalis* 24 h (□), 4 days (▨) and 8 days (■) after exposure to control (C) or conspecific alarm plus predator cues (A&T) in response to either control (C) or predator odour alone (T) during behavioural assays.

There was no antipredator response to alarm signals from crushed conspecifics alone (Fig. 1), as found in previous studies on gastropod molluscs (McCarthy & Dickey 2002; Ichinose et al. 2003). Alarm cues may not provide enough information about the type of risk to *L. stagnalis* to induce a response; alternatively they may not be perceived as being associated with a risk of predation at all. However, there was an increase in the proportion of individuals crawling out, as well as in the time spent out of the water when alarm cues were presented in combination with predator cues compared to the predator cue alone. This shows that alarm cues can be identified by *L. stagnalis*, and they probably increase the perceived risk in combination with predator cues compared to predator cues alone. An increase in antipredator response with perceived risk has been found in a number of aquatic species in relation to a predator threat, such as predator density (Ramcharan et al. 1992; Wiackowski & Staronska 1999), distance from prey (Turner & Montgomery 2003), number of prey consumed in the immediate environment (Van Buskirk & Arioli 2002), size of the predator (Kusch et al. 2004) and prey vulnerability (Alexander & Covich 1991; Ramcharan et al. 1992; DeWitt et al. 1999; Rundle & Brönmark 2001; Cotton et al. 2004). These factors may combine to increase the accuracy of the antipredator response in relation to the risk to the prey.

Cue Association

Experience of crushed conspecifics (alarm cues) plus predator odour from *T. tinca* induced an increase in antipredator behaviour on subsequent presentations of the *T. tinca* cue alone, equivalent to that seen in response to the predator plus alarm cues combined during the first experiment (Figs 1, 2). No significant increase in antipredator behaviour was seen in response to pre-exposure to alarm cues or predator cues alone prior to the behavioural assays. This suggests that *L. stagnalis* forms an association between the predator cue and an alarm substance, which then increases the perceived risk associated with *T. tinca* kairomones on subsequent encounter. Cue association between predator kairomones and damaged conspecifics may provide a more accurate assessment of potential risk from a predator, because it gives information about the current diet of that predator. For instance, whelks, *B. undatum*, increased their escape response over a period of 4 days exposure to a starfish, *Leptasterias polaris*, feeding on whelks, compared to exposure to a control or to *L. polaris* cues alone (Rochette et al. 1998). The juvenile *B. undatum* used were wild caught, so in this case, it is impossible to determine whether there was an innate response to the predator cue. It is likely that they had encountered *L. polaris* in the field; hence the laboratory experiment was potentially reinforcing previous experience.

Cue association may be necessary for a prey species to recognize the threat of predation accurately. The flatworm *D. dorotocephala* shows antipredator behaviour in response to injured conspecifics, but not to a potential predatory fish *Lepomis macrochirus* (Wisenden & Millard 2001). However, after exposure to damaged conspecifics plus *L. macrochirus* odour, *D. dorotocephala* showed antipredator behaviour in response to *L. macrochirus* odour alone 2 days later. As *L. stagnalis* responded to predator cues to a lesser extent without cue association being necessary, it seems likely that this population relies on cue association to give current information on the risk of predation rather than to identify the predator per se.

Cue association is an integral part of predator defence in most fish species studied (Utne & Bacchi 1997; Mirza & Chivers 2001; Brown 2003; Kelley & Magurran 2003; Larson & McCormick 2005). However, Arctic charr, *Salvelinus alpinus*, show innate as well as learnt recognition of potential predators (Vilhunen & Hirvonen 2003). Innate recognition of predators has also been found in a gastropod, *Physella virgata*, which changed its morphology in response to cues from six sunfish species (Langerhans & DeWitt 2002). These changes in morphology included a reduction in growth and a more rotund shape in the snails exposed to predator cues, which were thought to be in part the result of changes in behaviour. However, the snails were unable to respond differentially to predators in relation to their potential risk, as they were unable to differentiate between molluscivorous and nonmolluscivorous species.

Other studies that have found a response to predator cues alone usually concern wild-caught animals, where the history of chemical exposure is unknown, or the animals respond to a predator known to be present in their environment (Hopper 2001; Dahl & Peckarsky 2002; Åbjörnsson et al. 2004; Keppel & Scrosati 2004). As many species are capable of learning about potential predators, observed responses may be the result of experience.

Persistence of Cue Association

Cue association appeared strongest at 24 h postexposure, and showed a slight decline over time from 24 h to 8 days postexposure. At 8 days postexposure, the proportion of time spent crawled out was still significantly greater in the group previously exposed to predator plus alarm cues, suggesting that memory of the learned cue association persists for at least 8 days after exposure. Although the encounter rate between *T. tinca* and *L. stagnalis* in the source population is unknown, it seems feasible that 8 days is an ecologically relevant timescale in the field. The response to pumpkinseed fish, *L. gibbosus*, by the snail *Physa acuta* declined with both distance from the predator and age of the cue (Turner & Montgomery 2003). However, it is not known whether the decline with age of the cue, with a mean behavioural lifetime of 41 h, was due to an increase in perceived time since a predation event or to a decline in cue concentration per se. The decline over time in antipredator response by *L. stagnalis* implies a reduction in the perceived risk from *T. tinca* with time since experience of predation cues. This is potentially caused by the simulated rate of predation on conspecifics affecting the risk perceived by *L. stagnalis* (Van Buskirk 2002).

The ability to learn about potential predators may be important for a species responding to invasive predators or to a new predator regime if the prey species is expanding its range or simply when a predator has not been previously encountered. The crayfish *Orconectus virilis*, *Procambrus clarkii*, *O. rusticus* and *Austropatmobius pallipes* were able to form cue association between a pseudopredator, the common goldfish, *Carassius auratus*, and damaged conspecifics (Hazlett et al. 2002). The duration of this cue association varied between species, but in *P. clarkii* was still present after 3 weeks. Individuals that had not been exposed to the *C. auratus* cue in combination with damaged conspecifics showed no antipredator response to the *C. auratus* cue on subsequent exposure.

A response caused by a random pairing of unrelated cues inducing antipredator behaviour could prove very costly to prey species. Potentially, this is why an antipredator response declines over time, when the cue association is not reinforced. There is also the potential to prevent cue association through learned irrelevance, where cues are presented in a random order so that the organism learns that they are not associated before cues are presented together (Baker et al. 2003; Hazlett 2003), although so far there is no evidence that this would occur in response to a natural predator.

Lymnaea stagnalis has been used in studies to show the neuronal basis of cue association learning (Ito et al. 1999; Benjamin et al. 2000), although with highly artificial stimuli rather than predator cues occurring in the natural environment, and to examine the effects of predator cues on antipredator behaviour and phenotypic plasticity (Rundle & Brönmark 2001; Rundle et al. 2004); however, ours is the first study to show the ability of a freshwater gastropod to learn about predation risk. Our results concur with those of Rochette et al. (1998) who examined the response of a marine gastropod to cue association, although their study did not consider the effect of prior exposure to alarm cues alone or the experience of their wild-caught snails. Our data therefore indicate that the mechanism of cue association learning may be similar in these diverse gastropod taxa.

Cue association learning, combined with variation in response to different types of cue, as shown in this study, could allow individuals to assess real and immediate risks from potential predator species in their environment. The observed decline in response with lack of reinforcement would also allow organisms to show avoidance behaviours only in the face of active predators and high predation risk, and to reduce costs associated with unnecessary escape behaviour. Such fine tuning of behavioural responses to environmental cues may be highly adaptive, and the precise mechanisms involved represent an intriguing area for future research.

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Predator regime influences innate anti-predator behaviour in the freshwater gastropod *Lymnaea stagnalis*

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SUMMARY

1. Predation incurs high fitness costs in aquatic organisms either through direct consumption or through avoidance responses that reduce time for activities such as feeding and reproduction. Hence, avoidance responses of aquatic organisms should vary to match closely the predation threat in their environment.

2. The freshwater gastropod *Lymnaea stagnalis* occurs in a variety of environments which vary in the presence or absence of predatory fish. We used naïve snails reared from six populations of this species experiencing different predator regimes (three co-occurring with molluscivorous fish and three without) to assess whether populations differed in the type and degree of their avoidance behaviours. Innate behavioural responses to four treatments (control, conspecific alarm cues, fish kairomones and fish kairomones paired with alarm cue) were compared in laboratory trials.

3. The primary anti-predator behaviour of *L. stagnalis* in response to fish kairomones was to crawl out of the water rather than seek refuge under water. This response was strongest when fish kairomones were paired with alarm cues, and varied depending on population origin; snails reared from populations co-occurring with predatory fish showed a stronger response than those raised from populations not experiencing such predators. In addition, populations co-occurring with predatory fish responded to the fish kairomones presented alone.

4. Our findings suggest that the degree of innate anti-predator behaviour shown by *L. stagnalis*, in terms of both the level of risk to which it responds and the degree of response, varies depending on the predator regime experienced by field populations. Together with previous work on cue association, this demonstrates that this gastropod is able to match its avoidance behaviour very closely to short and long term predation threats within its habitat.

Keywords: alarm cue, chemical communication, gastropod, induced defences, local adaptation

Introduction

Predators can impose high fitness costs on their prey species, directly through predation events but also indirectly, either by modifying prey behaviour so as to reduce opportunities to feed or reproduce (Lima & Dill, 1990) or by imposing costs through plastic morphological responses (DeWitt, 1998; Caramujo & Boavida, 2000; Van Buskirk, 2000). Changes in morphology and behaviour in response to one predator may also make prey organisms more vulnerable to alternative predator species (Sih, Englund & Wooster, 1998). A number of studies have demonstrated strong responses to predation cues in freshwater systems (Reviewed in: Dodson *et al.*, 1994; Chivers & Smith, 1998; Lass & Spaak, 2003; Werner & Peacor, 2003). However, many aquatic prey species occupy a wide variety of habitats and experience varying levels of predation

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threat, such that a single mode of behavioural response is unlikely to be an effective method of avoiding all potential predators (McPeek, 1990). Hence anti-predator behaviour should vary between populations with different predator regimes to match closely the type and level of predation threat present.

Local adaptation to predation threat is likely to occur in relatively sedentary species with low dispersal abilities, whereas induced responses are more likely in species which have potential for high dispersal between generations, when parents and offspring are more likely to encounter alternate predator regimes (Kawecki & Ebert, 2004). Adaptation may also be favoured where plastic responses are costly if the lag between expressing one phenotype or its alternative is too far behind the changes in the environment (Padilla & Adolph, 1996), where information gathering to produce the correct phenotype is costly (DeWitt, 1998) or where costly responses to non-threatening species are likely (Langerhans & DeWitt, 2002).

Gastropods have been utilized extensively to study the responses to predation threat in aquatic systems. Freshwater gastropods have limited dispersal ability, as they are only able to spend short periods emersed without risking desiccation. As such it may be predicted that populations will be under selection dependent on the predator regime they experience, and this prediction is supported by empirical data (Covich *et al.*, 1994; McCarthy & Fisher, 2000; Bernot & Whittinghill, 2003). However, these studies have not explicitly tested for differences between populations across replicate sites differing in their predator status. Also, this previous work used wild-caught animals, which means that any differences between populations may reflect plastic responses rather than local adaptation. Previous studies using laboratory reared animals suggest that the anti-predator response in freshwater gastropods does have an innate element, but can also vary with experience (Dalesman *et al.*, 2006; Turner, Turner & Lappi, 2006).

The great pond snail, *Lymnaea stagnalis* (Linnaeus, 1758), inhabits a wide range of freshwater habitats, including those with and without predatory fish and might be predicted to exhibit adaptation in response to these differing predator regimes. This species has been demonstrated to have both innate and learnt components in its behavioural response to predator cues (Dalesman *et al.*, 2006). Here, we investigate whether the innate aspects of this behaviour varies between populations experiencing differing predator regimes. F1-generation snails were tested for their responses to cues from tench, *Tinca tinca* (Linnaeus, 1758), a known gastropod predator (Brönmark, 1994) that commonly overlaps in distribution with *L. stagnalis*. Responses to fish kairomones were tested both on their own and concurrently with cues from crushed gastropods to simulate a predation event. Hence, we were able to assess how populations differed in their response to the type of cue presented and in the degree of response. Adaptation through natural selection has been shown to account for divergence between aquatic populations experiencing differing predation pressure (Cousyn *et al.*, 2001; O'Steen, Cullum & Bennett, 2002; Meyer *et al.*, 2006). As such we predicted that populations that co-existed with predatory fish would have adapted to show higher innate responses than those that did not experience such predation risk.

Methods

Field collection sites

Adult *L. stagnalis* used to produce experimental snails were collected between September 2005 and May 2006 from six sites on the Somerset Levels, an extensive wet meadow system in the southwest U.K., covered by a series of interlocking drainage ditches and channels. The three sites with predatory fish present (South Drain – 51.18N 2.88W, Sowy River – 51.07N 2.88W, North Moor – 51.07N 2.96W) were large channels (width: 6–22 m, depth: 0.7–1.1 m). Information from the local angling club, Taunton Angling Association (S. Dalesman, pers. com.), was used to confirm the presence of tench at these sites. The three other sites (Chilton Moor – 51.19N 2.88W, Little Hook – 51.06N 2.87W, Wistaria Farm – 51.07N 2.98W) were small ditches (width: 1.8–2.9 m, depth: 0.45–0.7 m); electrofishing was used to confirm the absence of molluscivorous fish. Sites with and without predatory fish were located in a pair-wise fashion such that each site lacking predatory fish was closer to a site with predatory fish than to the nearest site without such fish and vice versa to reduce any potential influence of geographic location. The mean distance between sites with and without predatory fish was 960 m (range from 865–1036 m). Repeated sampling at all the sites between March 2004 and September 2006 indicated that *L. stagnalis* is common at these sites for most of the year.

Study organisms

In the laboratory, adult *L. stagnalis* were maintained in aquaria with 4 L of aerated artificial pond water (ATSM, 1980) with 90 mg L^{-1} [Ca^{2+}] (Rundle *et al.*, 2004) at 15 ± 1 °C under a 12 : 12 light : dark cycle, and fed on Iceberg lettuce and spinach. A minimum of 12 adults were collected from each source population to breed the F1 generation to ensure juveniles used for behavioural trials were representative of the population of origin. To reduce the influence of adult experience on juvenile development and behaviour, the F1 snails (6 ± 0.5 mm spire height) used as responders were bred from egg masses laid a minimum of 2 months post collection. They were then maintained separately in the same conditions as the adults. Tench used to produce predator cue were kept in analogous condition to the snails, and fed Nutri-flake (Hozelock Cyprio, Birmingham, U.K.), which contains no mollusc extracts, and hence avoided potential dietary cues affecting the trials.

Odour production

Predator kairomones and alarm cues were produced in the same way in all the experiments (Dalesman *et al.*, 2006). Four exposure treatments were used: (i) fish kairomone water, produced by placing three tench (length 10 ± 1 cm) into 4 L of aerated artificial pond water for 1 h; (ii) alarm cue, produced by mixing three crushed snails (6 ± 0.5 mm) into 4 L of aerated artificial pond water; (iii) fish kairomone plus alarm cue, produced by mixing three crushed snails (6 ± 0.5 mm) into 4 L of tench cue water; and (iv) the control which was aerated artificial pond water. During a single behavioural trial six individuals were exposed to each of the four treatments and the same odour treatment was used for all six individuals (i.e. tench odour was only produced once for each behavioural trial). To complete each treatment × population combination four behavioural trials were required, and odour treatments were produced separately for each trial.

Behavioural assay

Behavioural trials were carried out in the same way for all experiments under laboratory conditions analogous to those in which the snails were maintained (Dalesman *et al.*, 2006). Twenty-four individuals were used for each treatment combination, giving a total of 96 individuals per population, 576 in total, with no individual snail used more than once during behavioural trials. Behavioural chambers consisted of a white plastic dish, 165 mm diameter × 60 mm depth (A.W. Gregory & Co. Ltd., London, U.K.), with a longitudinally sectioned white PVC pipe (36 mm long, 30 mm diameter) attached open side down to the centre using non-toxic sealant (Wickes Ultimate Sealant and Adhesive; Wickes Building Supplies Ltd., Northampton, U.K.) to provide a refuge. Juvenile snails were acclimated to behavioural chambers for 24 h in 630 mL of artificial pond water prior to behavioural assays. Seventy millilitres of cue water was added to each chamber at the start of the behavioural assay to give a final concentration of 10% cue water. The position of each snail within the behavioural chambers was recorded every 5 min for 2 h following cue addition. Crawl-out behaviour has been found to be the main anti-predator response to tench cue by *L. stagnalis* originating from a population with fish predators present (Dalesman *et al.*, 2006). However, use of a refuge by *L. stagnalis* may occur in response to fish predators (Rundle & Brönmark, 2001), so both types of behaviour were analysed here using two variables: the proportion of total time spent in avoidance behaviour; and the latency to avoidance, calculated as the proportion of total time during the trial before an avoidance behaviour was observed (Cotton, Rundle & Smith, 2004).

Statistical analysis

Data were analysed using ANOVA in GMAV5 (Institute of Marine Ecology, University of Sydney, Sydney, Australia). Initially each population was analysed separately to test for an effect of trial on the response to alarm and tench cues, however as no effect of trial was found in any of the six populations trial was excluded from further analyses. Alarm cue, tench cue, habitat type (predatory fish present or absent) and population (nested within habitat type) were used as factors in the analysis. Both time spent in avoidance and latency to avoidance were calculated as proportions of total duration, and were arcsine-square root transformed prior to analysis (Sokal & Rohlf, 1995). Student–Newman–Keuls (SNK) tests were used to carry out *post hoc* pair-wise comparisons.

Results

The treatment with a combination of tench kairomones plus alarm cue produced the greatest avoidance response, with the longest time spent crawled out for all populations. This response was significantly higher for individuals bred from adults that originated from populations experiencing fish predation (Fig. 1a, Table 1; $F_{1,4} = 16.66$; $P = 0.015$; SNK; $P < 0.01$). In addition, snails originating from populations experiencing fish predation also showed a significant response to the fish cue treatment, though this was significantly lower than the response to combined cues (SNK; $P < 0.05$); individuals from populations where no fish predators were present showed no significant crawl-out response to fish cues alone. None of the populations showed a significant crawl-out response to alarm cues in isolation.

The latency to crawl-out was significantly shorter in response to tench kairomones and alarm cues combined ($F_{1,4} = 194.13$; $P < 0.001$) compared with either cue presented alone for all the populations tested, and did not differ depending on habitat origin (Fig. 1b; Table 1). However, the response to tench kairomones presented alone differed significantly between habitats, with latency being significantly lower in populations originating from areas with fish predators present (Fig. 1b; Table 1; $F_{1,4} = 76.08$; $P = 0.001$; SNK; $P < 0.01$).

The time spent under the refuge significantly decreased in response to the presence of tench cue (ANOVA: main effect of tench cue; $F_{1,4} = 13.90$; $P = 0.020$) but was not affected by any other factors.

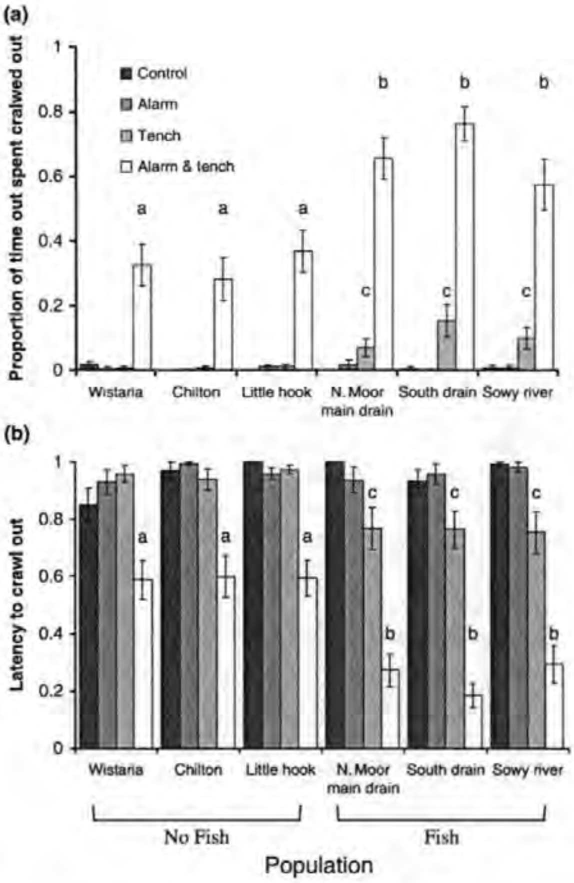

Fig. 1 The mean (±SE) crawl out response of F1 *L. stagnalis* from populations with low (Wistaria; Chilton; Little Hook) and high (N. Moor Main Drain; South Drain; Sowy River) predatory fish regimes, showing (a) proportion of time spent crawled out and (b) latency to crawl out under different treatments ($n = 24$ for each treatment combination). Columns without letters do not differ significantly from one-another but differ significantly from all columns with letters. Columns with the same letter do not differ significantly, but do differ from columns bearing alternative letters.

Discussion

All the populations used in this study showed a degree of innate anti-predator response to predator kairomones presented in combination with alarm cues by crawling above the water line. This indicates that the populations not experiencing fish predation are still able to recognize the predation threat from tench and respond with appropriate behaviour. The response from fishless populations was significantly lower than from populations exposed to predatory fish, however, suggesting that the innate perceived threat is lower in the fishless populations. The populations from habitats with predatory fish present also demonstrated an innate response to tench cues alone, not seen in the populations without any predatory fish in their natural habitat. The observed decrease in use of the refuge in response to tench cues is most likely to be an artefact of the increase in crawl out response to this cue, confirming that crawl out behaviour is the main response to tench kairomones

Table 1 Results from four-way nested ANOVA on time spent crawled out of the water and latency to crawl out. Factors used are: habitat type (predatory fish/no predatory fish), population (nested in habitat type), alarm cue (present/absent) and tench cue (present/absent). $n = 24$ for all treatment combinations

		Proportion of time			Latency		
Source	d.f.	Mean square	*F*	*P*-value	Mean square	*F*	*P*-value
Habitat type (Ha)	1	3.41	27.76	0.006	4.10	30.61	0.005
Population nested in habitat (Po)	4	0.12	1.94	0.103	0.13	1.27	0.282
Alarm cue (A)	1	14.61	266.87	<0.001	13.91	248.47	<0.001
Tench cue (T)	1	24.62	222.23	<0.001	31.16	416.26	<0.001
Ha*A	1	0.73	13.27	0.022	0.35	6.25	0.067
Ha*T	1	3.72	33.57	0.004	5.70	76.08	0.001
A*Po	4	0.05	0.86	0.486	0.06	0.53	0.714
T*Po	4	0.11	1.75	0.138	0.07	0.71	0.586
A*T	1	14.24	355.51	<0.001	14.05	194.13	<0.001
Ha*A*T	1	0.67	16.66	0.015	0.13	1.80	0.251
A*T*Po	4	0.04	0.63	0.640	0.07	0.69	0.602
Total	575						

shown by *L. stagnalis*, as found in a previous study (Dalesman *et al.*, 2006).

The difference between high and low risk populations in a laboratory-reared F1 generation suggests that adaptation to local predator regimes has occurred in the six populations studied. This result concurs with results on responses from other freshwater organisms with limited dispersal such as *Gammarus pulex* (Åbjörnsson, Hansson & Brönmark, 2004) and the Trinidadian guppy, *Poecilia reticula* (O'Steen *et al.*, 2002), which also showed divergence in the degree of innate response to predatory fish cues relative to the predators present in their environment. As all three of these species have relatively low dispersal abilities these results support the theory that species with low dispersal capabilities adapt to local predator regimes rather than relying purely on plasticity to alter phenotype (Kawecki & Ebert, 2004).

The reduced response to fish cues from populations not normally exposed to predatory fish suggests that *L. stagnalis* does not have a general anti-predator response to all the potential predator species it could encounter, as these populations showing low responses to predatory fish cues are exposed to large numbers of invertebrate predators (S. Dalesman, pers. obs.) whose numbers would be reduced by predatory fish elsewhere (Åbjörnsson, Brönmark & Hansson, 2002). Indeed, it has been previously demonstrated that aquatic gastropods are able to vary their response depending on predator identity (Snyder, 1967; Turner, Bernot & Boes, 2000), so potentially populations may lose their response to one type of predator without reducing their response to another (Mikolajewski *et al.*, 2006). This may eventually result in the complete loss of the ability to recognize potential predator by a prey species if they no-longer come into contact (Stoks, McPeek & Mitchell, 2003). The reduced avoidance response in low risk populations suggests that there may be costs associated with retaining anti-predator behaviour towards fish. Traits linked with an increased tendency to respond to fish cues may incur costs when expressed in fishless environments, for example the loss of sexual ornamentation (Magurran, 1999; Basolo & Wagner, 2004) or the costs associated with maintaining and producing plastic responses (DeWitt, 1998).

Assuming that costs are associated with maintaining the high degree of anti-predator behaviour in response to fish cues when predatory fish are not present, it seems perplexing that the response is retained at all in the low risk populations studied here. One explanation may be that fishless environments can be invaded by predatory fish during times of flooding, as the fish and fishless populations used here are in relatively close proximity (<1.1 km) and on a flood plain. The high and low response populations are connected by a series of drainage ditches so there is also potential for gene flow between populations, preventing complete loss of anti-predator behaviour from the fishless populations. As the fitness consequences of not responding to fish correctly are likely to be much higher than occasional inappropriate behaviour when fish are not present, it is possible that even infrequent encounters with predatory fish may result in the behaviour being retained. An alternative explanation would be that at least part of the observed response to predatory fish cues is due to a general sensitivity to predators rather than a predator specific response, or linked within a suite of behavioural syndromes (Sih, Bell & Johnson, 2004). In this case the 'low risk' populations are still exposed to an assortment of invertebrate predators including beetles, bugs and leeches (S. Dalesman, pers. obs.), and elements of the response seen to fish kairomones may be included in the anti-predator response to these other taxa.

The nature of the environment in which the snail populations used in this study live, with the close proximity and potential for migration along linked water systems suggests that the differences seen will be under strong selection, both for high response under threat of predation, but also to lose responses when that threat is no longer present. The degree of variation seen in both population groups, with a few individuals from the populations lacking predatory fish showing a high response to fish and alarm cues, and vice versa, indicates potential within the population to react rapidly to a change in predatory threat. Rapid microevolution causing divergence between conspecific populations (Hendry & Kinnison, 1999) has been demonstrated elsewhere as a result of natural selection caused by predators (Cousyn *et al.*, 2001; O'Steen *et al.*, 2002; Losos, Schoener & Spiller, 2004; Meyer *et al.*, 2006; Nosil & Crespi, 2006).

In conclusion, our study suggests that innate differences in behaviour and morphology found in other aquatic species (O'Steen *et al.*, 2002; Åbjörnsson *et al.*, 2004) may be generalized across freshwater taxa with poor dispersal abilities. The inability to move from areas of high predation risk to low predation risk may be promoting adaptive responses to local predator regimes. We have previously demonstrated that *L. stagnalis* is able to alter risk perception of a known predator based on recent experience (Dalesman *et al.*, 2006). Such abilities to associate predation cues with perceived risk to fine-tune the innate recognition of predation threat in the short term, alongside the long term adaptive response to predators demonstrated in the present study, suggest that freshwater snails such as *L. stagnalis* are able to match their anti-predator behaviour closely to their environment.

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PHYLOGENETIC RELATEDNESS AND ECOLOGICAL INTERACTIONS DETERMINE ANTIPREDATOR BEHAVIOR

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Abstract. Interspecific recognition of alarm cues among guild members through "eavesdropping" may allow prey to fine-tune antipredator responses. This process may be linked to taxonomic relatedness, but might also be influenced by local adaptation to recognize alarm cues from sympatric species. We tested this hypothesis using antipredator responses of a freshwater gastropod *Lymnaea stagnalis* (L.) to alarm cues from damaged conspecific and 10 heterospecific gastropod species. As predicted, the magnitude of antipredator response decreased significantly with increasing phylogenetic distance, but increased when species were naturally sympatric (defined as species cohabiting in the same water body) with the source population of *L. stagnalis*. The responses to sympatric species were higher overall, and the relationship between genetic distance and alarm cue response was stronger when tested with sympatric species. This is the first study to demonstrate that population sympatry influences innate antipredator responses to alarm cues from intraguild members and suggests that responses based on phylogenetic relationships can be modified through local adaptation. Such adaptation to heterospecific alarm cues suggests that species could be at a disadvantage when they encounter novel intraguild members resulting from species invasion or range expansion due to a reduction in the presence of reliable information about predation risk.

Key words: alarm cue; chemical communication; gastropod; induced defenses; local adaptation; phylogeny.

Introduction

Biological signals produced by organisms are generally intended to communicate only with conspecific individuals conferring benefit to the signaler, but may be intercepted by heterospecific individuals through "eavesdropping" (Stowe et al. 1995, Bradbury and Vehrencamp 1998). Eavesdropping implies no benefit to the signaler, but may be of benefit to the receiver, for example in locating resources or identifying potential competitors, and has been found to be important in a wide variety of taxa (Catchpole and Leisler 1986, Stowe et al. 1995, Taga and Bassler 2003, Symonds and Wertheim 2005, Runyon et al. 2006). Eavesdropping on alarm cues from heterospecific intraguild members has been found in several species and may be used to provide additional information about potential predation risk (Stenzler and Atema 1977, Mirza and Chivers 2001, Hazlett and McLay 2005, Schoeppner and Relyea 2005).

In communication systems where it is beneficial to both signaler and receiver to respond to related heterospecifics, phylogenetic relationships generally appear to explain the patterns observed, for example, the similarity of chemical aggregation signals in closely related *Drosophila* spp. (Symonds and Wertheim 2005) or attraction of *Aplysia* spp. to heterospecifics during egg laying (Cummins et al. 2005). In these cases, there is a benefit to all individuals to oviposit in the same site, irrespective of their species. The interaction between evolutionary history and local adaptation in shaping interspecific communication has also been investigated in relation to sexual signals, where it has been shown that differences between signaling systems are more pronounced in sympatric species pairs (Ryan and Rand 1995, Symonds and Elgar 2004). Phylogeny is not a good predictor of the degree of divergence seen between sexual signals when species are found sympatrically, though exceptions have been found (Smith and Florentino 2004). The importance of sympatry in shaping sexual signals suggests that local adaptation to avoid heterospecific interactions is acting on this signaling system, and in many cases counteracting the effects of phylogenetic relatedness.

In predator–prey systems, recognition of alarm cues from closely related species is not considered communication as there is no benefit to the signaler (Bradbury and Vehrencamp 1998), however it may be of benefit to the receiver to assess predation risk accurately and, hence, may reduce the high fitness costs associated with predation (Lima and Dill 1990). There is considerable evidence that alarm cues are conserved within phylogenetic groups (Pfeiffer 1977), with responses to alarm cues from closely related species generally being stronger than from those more distantly related (Snyder 1967, Stenzler and Atema 1977, Brown et al. 2003, Vilhunen

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and Hirvonen 2003), though the nature of the relationship between phylogenetic distance and response has only been investigated for the tadpole *Hyla versicolor* (Schoeppner and Relyea 2005). Snyder (1967) proposed that responses to alarm cues from heterospecific species sharing a common predator would be advantageous to a prey species regardless of taxonomic relationships. This theory would suggest that the effect of phylogenetic relatedness might be modified via the effects of sympatry or allopatry. There have been several studies which suggest that sympatry may modify responses to alarm cues from heterospecific species, however, they either used wild caught individuals so could not rule out the effects of experience (Stenzler and Atema 1977, Chivers et al. 1997, Rochette and Dill 2000, Sullivan et al. 2003, Hazlett and McLay 2005) or used too few species to investigate any interaction with the effects of phylogenetic relatedness (Laforsch et al. 2006).

In aquatic systems, chemical cues often provide the most reliable source of information about a predator where other information, such as visual cues, may be impaired by poor visibility (Dodson et al. 1994). Information from predator diet or injured conspecifics can be used to assess current predator feeding activity and hence associated risk from a potential predator (Dalesman et al. 2006), or to learn to identify a previously unrecognized predation threat (Chivers and Smith 1998). Due to selective forces operating on prey species to respond correctly to predation threat it seems likely that the relationship between phylogenetic distance of intraguild members from a responder and antipredator response to alarms cues will be modified by local adaptation.

Here we investigated the innate response of a freshwater gastropod, *Lymnaea stagnalis* (L.) to alarm cues from heterospecific prey guild members, presented with cues from a fish, (tench, *Tinca tinca* L.) known to feed on gastropods (Brönmark 1994). We used 1000 heterospecific freshwater gastropods at differing phylogenetic distance from *L. stagnalis*, five of which are found sympatrically to the source population of *L. stagnalis* used during behavioral trials, and five of which are allopatrically distributed. In this case, species were considered allopatric if they were not found in the same body of water (South Drain) as the *L. stagnalis* population (S. Dalesman, *unpublished data*), allopatric species in some cases came from a site less than 1 km away. We tested the following hypotheses: (1) that the innate antipredator response of *L. stagnalis* to alarm cue, paired with predatory fish cues, would decrease with increasing phylogenetic distance of the heterospecific snail species used to produce alarm cue; and (2) that the response to alarm cue from species found sympatrically to the *L. stagnalis* population would induce stronger antipredator behavior than alarm cue from species found allopatrically distributed relative to the *L. stagnalis* population.

METHODS

Study organisms.—Adult *L. stagnalis* used to establish the snail culture for behavioral trials were collected from South Drain, a drainage canal on the Somerset Levels, UK (51°10′48″ N, 2°52′48″ W) in September 2005. Repeated field sampling between June 2004 and June 2006 identified which gastropod species were present at the South Drain site. In the laboratory, adults were kept in aquaria with 4 L of aerated artificial pond water (American Society for Testing and Materials 1980) with 90 mg/L [Ca2+] (Rundle et al. 2004). They were maintained at 15° ± 1°C under a 12 h:12 h light:dark cycle, and fed on iceberg lettuce and spinach. To minimize the influence of adult experience on juvenile development and behavior, the F1 snails used during behavioral trials were bred from egg masses laid a minimum of two months post collection and were then maintained separately in the same conditions as the adults. Heterospecific adult snails were collected from several locations (Appendix A) to raise F1 snails that were crushed to produce alarm cue. All snails used had a standard spire height of 6 ± 0.5 mm except in the case of ramshorn species (*Planorbis planorbis* and *Planorbarius corneus*) where spiral diameter measured 6 ± 0.5 mm.

Genetic distance between species was calculated using a 370 base pair section of 18S rDNA obtained from Genbank (Appendix A). Sequences were aligned using ClustalX 1.83 (Thompson et al. 1997) and a pairwise distance matrix calculated in TREECON 3.0 (Van de Peer and De Wachter 1993) using the Kimura two-parameter model (Kimura 1980), with the transition/transversion ratio estimated from the data and insertions/deletions taken into account. As no 18S rDNA sequence is currently available for *Lymnaea fusca*, the sequence for *L. palustris* was used as a closely related species within the sub-genus *Stagnicola*; *Lymnaea palustris* is the only other British species found in the subgenus (Anderson 2005), and is known to be very closely related to *L. fusca* based on ITS-2 sequence data (Bargues et al. 2001).

Odor production.—Predator and alarm cues were produced in the same way in all the experiments (Dalesman et al. 2006). Tench, *Tinca tinca*, were used to produce predator cue as they are a known natural predator of freshwater gastropods (Brönmark 1994). The tench were fed Nutri-Flake (Hozelock Cyprio, Aylesbury, UK), which contains no mollusk extracts, and hence avoided potential dietary cues affecting the trials. Predator cue water was produced by placing three tench (length 10 ± 1 cm) into 4 L of aerated artificial pond water for one hour. Alarm cue was produced by mixing three crushed snails (6 ± 0.5 mm) into 4 L of aerated artificial pond water. Predator plus alarm cue water was produced by mixing three crushed snails (6 ± 0.5 mm) into 4 L of tench cue water. The control was the addition of aerated artificial pond water with no cues added. Crushed snails were used as opposed to feeding snails to tench to ensure that the alarm cue concentration paired with tench cue was kept at a similar level for all behavioral trials, the strong behavioral response in the paired cue treatment in a previous study suggested that *L. stagnalis* responds to alarm cue from crushed snails paired with tench cues as a predation threat (Dalesman et al. 2006).

Behavioral assay.—Behavioral trials were carried out between January and May 2006 in the Experimental Aquatic Ecology Laboratory, University of Plymouth in conditions analogous to those in which the snails were maintained (Dalesman et al. 2006). Twenty-four individuals were used for each treatment combination, with each snail used once only, giving a total of 1056 snails. Behavioral chambers consisted of a white plastic dish, 165 mm diameter × 60 mm depth (A. W. Gregory and Co., London, UK), with a longitudinally sectioned white PVC pipe, 36 mm long, 30 mm diameter, attached open side down to the centre using nontoxic sealant (Wickes Ultimate Sealant and Adhesive, Wickes Building Supplies Ltd., Middlesex, UK) to provide a refuge. Twenty-four chambers were set up for each behavioral trial, and juvenile snails were acclimated to behavioral chambers for 24 hours in 630 mL of artificial pond water prior to behavioral assays. Cue water (70 mL) was added to each chamber at the start of the behavioral assay to give a final concentration of 10% cue water, with six chambers having each of the four cue treatments added on each day. To avoid cross-contamination, alarm cue from a single species was used on each day, resulting in four behavioral trials in total for each snail species. The position of the chambers in which each treatment level was added was fully randomized within the 24 chambers for each behavioral trial. The position of each snail within the behavioral chambers was recorded every 5 minutes for 2 hours following cue addition. Crawl-out behavior is the main antipredator response of *L. stagnalis* to tench (Dalesman et al. 2006), hence only results from crawl-out behavior are presented here. Crawl-out behavior was analyzed using two variables: the proportion of total time spent crawled out of the water; and the latency to crawl out, calculated as the proportion of total time during the trial before crawl-out behavior was observed (Cotton et al. 2004).

Statistical analysis.—Data were analyzed as an unbalanced ANCOVA design using General Linear Model (GLM) in Minitab 13 (Minitab, State College, Pennsylvania, USA). Alarm cue, tench cue and environment (whether snails were found sympatrically or allopatrically) were treated as fixed factors, and pairwise genetic distance between *L. stagnalis* and each of the species used to produce alarm cue was used as the covariate. Both time spent crawled out and latency to crawl out were calculated as proportions of total duration, and were arcsine-square root transformed prior to analysis (Sokal and Rohlf 1995). Post hoc tests on response means versus genetic distance were carried out using a regression analysis in Minitab13.

RESULTS

The principal antipredator behavior of *Lymnaea stagnalis*, crawling above the water line, was greatest in response to alarm and tench cue combined, both in the mean proportion of time spent out of the water and the mean latency to crawl out. The effects of genetic distance or sympatry/allopatry were not seen in response to the control or either cue presented alone. However, in response to combined cues (alarm and tench cue) the proportion of time spent crawled out declined significantly with genetic distance between *L. stagnalis* and the heterospecific snail. This trend was modified depending on whether the snail species used is found allopatrically or sympatrically to the responding population of *L. stagnalis*; the response to allopatric species was significantly lower than that to sympatric species, with a weaker decline with increasing genetic distance (Fig. 1a; ANCOVA, four-way interaction: $F_{1,1040} = 4.84$, $P = 0.028$; Appendix B). In response to combined cues the latency to crawl out was significantly longer with increased genetic distance; this response was again modified by whether heterospecifics used to produce alarm cue were found sympatrically to the *L. stagnalis* population, with a significantly shorter latency to crawl out when crushed snails were found in sympatry (Fig. 1b; ANCOVA, four-way interaction, $F_{1,1040} = 3.95$, $P = 0.047$; Appendix B).

Post hoc analysis of the response to combined cues (alarm and tench cue together) showed that when alarm cue was produced by sympatric species there was a highly significant relationship between genetic distance and strength of the response for both the proportion of time crawled out (Fig. 1a; R^2(adj) = 0.90, $P = 0.003$) and latency to crawl out (Fig. 1b; R^2(adj) = 0.94, $P = 0.001$). However, this relationship was not apparent when the species used to produce alarm cue were allopatric, with a weaker non-significant relationship between genetic distance and proportion of time spent crawled out (Fig. 1a; R^2(adj) = 0.63, $P = 0.070$), and no apparent relationship between genetic distance and latency to crawl out (Fig. 1b; R^2(adj) = 0.00, $P = 0.396$).

DISCUSSION

This study demonstrates that the magnitude of innate avoidance responses shown by *Lymnaea stagnalis* to heterospecific alarm cues paired with predator cues, declines with phylogenetic distance. This declining response concurs with findings on innate tadpole behavior (*Hyla versicolor*) where timing of evolutionary divergence was compared with response level (Schoeppner and Relyea 2005). Although the relationship between genetic distance and antipredator response of *L. stagnalis* is very strong when the responding population is found sympatrically to the species used to produce alarm cues, this relationship is weaker and

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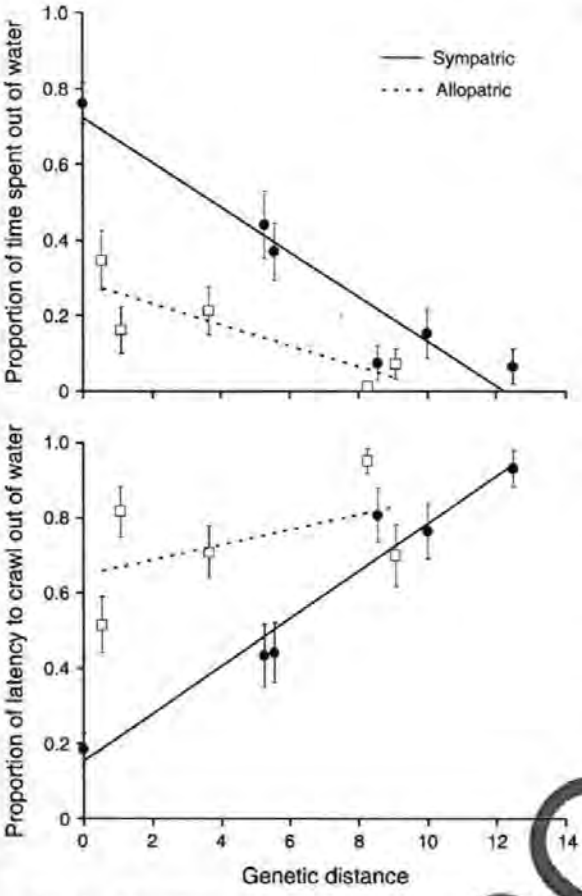

FIG. 1. The relationship between genetic relatedness and sympatry/allopatry in the antipredator response of *Lymnaea stagnalis* (a freshwater gastropod) to tench (*Tinca tinca*, a fish that feeds on gastropods) and alarm cues combined. Individual symbols show the response (mean ± SE) toward each species (solid circles, sympatric species; open squares, allopatric species), with lines indicating the trend of relationship between behavior and Kimura two-parameter genetic distance for (a) proportion of time spent crawled out of the water and (b) proportion of latency to crawl out. $N = 24$ snails for each data point.

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not significant when considering the response to allopatric species, particularly in the latency to crawl out. This is the first evidence to demonstrate that the effect of relatedness on prey responses to heterospecific alarm cues can be modified by ecological context and could disappear if the species used are allopatric to the responding population.

The agreement between our findings for sympatric species and those of other studies (Mirza and Chivers 2001, Schoeppner and Relyea 2005, Kelly et al. 2006) suggests that the influence of phylogenetic relatedness may indeed be widespread in chemical alarm signaling. This relationship between response and genetic distance indicates there may be a gradual mode of evolution in either the signaling chemicals or the way in which they are detected (Jovelin et al. 2003, Symonds and Wertheim 2005, Kelly et al. 2006). The complete lack of response by *L. stagnalis* to alarm cues presented in isolation alone concurs with previous findings for this species (Dalesman et al. 2006), and may reflect the general unreliability for prey species of using alarm cues alone, as it provides no information about the nature of the threat (Chivers and Smith 1998).

The significant effect of sympatry on intensifying antipredator response suggests that *L. stagnalis* has either conserved its response relative to allopatric species or elevated it toward sympatric species. This type of adjustment has been demonstrated previously in response to predator cues where individuals from environments with high numbers of predators show a greater innate response to predator cues than those from low risk environments (Boersma et al. 1998, Magurran 1999, Åbjörnsson et al. 2004), although this may be due to transgenerational effects as well as local adaptation (Agrawal et al. 1999). However, our study is the first to conclusively demonstrate that sympatry can affect the innate antipredator response to alarm cues from heterospecifics. Previous studies that inferred evidence for an effect of sympatry did not control adequately for either the effects of experience, by using wild caught individuals (Stenzler and Atema 1977, Chivers et al. 1997, Rochette and Dill 2000, Sullivan et al. 2003, Hazlett and McLay 2005), or used too few species to draw strong conclusions (Laforsch et al. 2006). Together these factors may account for the bias toward the importance of phylogenetic relationships affecting the response to alarm cues in the current literature.

Although tench are likely to represent the main predation threat to aquatic gastropods in the site where our responding population was sourced, it is likely that *L. stagnalis* and co-habiting gastropod species will encounter other predator threats. The presence of generalist predators or specialist predators that focus on the taxonomic group including the prey species of interest, is likely to increase selection to respond to heterospecific cues, whereas specialist predators that focus on other taxonomic groups would select against costly induced responses to heterospecific cues. The degree to which a taxonomic group is represented in the predator's diet will affect the degree to which a response to heterospecific alarm cues is appropriate. For example, both crayfish, *Pacifastacus leniusculus*, and tench, *Tinca tinca*, consume a large number of gastropods, so there is a high probability that when they are actively foraging in an area all gastropod species have a high risk of predation (Brönmark 1994, Nyström et al. 2001). However, if a rainbow trout (*Oncorhynchus mykiss*) which specializes on aquatic insects, happens to consume an aquatic gastropod, it is unlikely that they will pose a significant threat to other gastropods in the vicinity (Nyström et al. 2001). This may have two effects on the response to alarm cues from heterospecifics: (1) the response may be predator specific, such that the prey species only responds to heterospecific cues when paired with cues from specific predators', and (2) the selection

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to respond to heterospecific species may only occur when there are predators present that predate several species within a taxonomic group.

A general reliance on heterospecific cues to indicate the level of predation risk in the environment may be detrimental if invasive intraguild species move into the habitat whose alarm cues are not recognized. In this case, the responding species may under-estimate the risk of predation resulting in a reduced antipredator response and potentially increased vulnerability to predation. Likewise, if a species colonizes a novel habitat it may encounter new intraguild members. The phylogenetic relationship between the responding species and the novel intraguild members is likely to influence the potential for recognition of alarm cues as demonstrated in this study. Adaptive responses to a novel species appear to have occurred historically in the population of *L. stagnalis* used as responders. We found that *L. stagnalis* exhibits an elevated response to alarm cue from the invasive gastropod *Physella acuta*. This response to a sympatric introduced species is elevated relative to that for a closely related native British species, *Physa fontinalis* found allopatrically distributed relative to the South Drain *L. stagnalis* population.

Differences between populations in response to alarm cues have previously been suggested to occur through learning (Brown 2003), however there is good support for genetic differentiation in response to predators (Åbjörnsson et al. 2004, Kawecki and Ebert 2004) and it would therefore seem highly likely that prey species would also show adaptive variation in response to alarm cues. It appears, in our study, that individuals from the population used as responders have modified their response toward sympatric species. As our responders were naïve lab reared snails we are able to rule out effects of experience, which have been used previously to explain strong responses to sympatric species (Brown 2003). Here it is clear that local adaptation rather than behavioral plasticity is important. Our study demonstrates for the first time that sympatry can enhance innate responses to heterospecific alarm cues and that this process can interact with phylogenetic relationships to fine-tune antipredator behavior.

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APPENDIX A

Gastropod species used to produce alarm cue (*Ecological Archives* XXXX-XXX-XX).

APPENDIX B

Results from four-way ANCOVAs using genetic distance as the covariate (*Ecological Archives* XXXX-XXX-XX).