

LIFE SKILLS TRAINING IN HATCHERY REARED FISH

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Life skills training in hatchery reared fish

Thesis abstract

Releasing hatchery reared fish into wild habitats has become an increasingly employed strategy for bolstering conservation or commercially important fish stocks. This method, however, has often met with limited success and the hatchery fish suffer from a high rate of mortality upon release. Reasons for this failure are often attributed to the underdeveloped behavioural traits displayed by hatchery reared fish. The rearing of fish in an impoverished hatchery environment may not allow for the development of a full complement of behaviours required to survive the rigours of the natural environment. There is increasing interest in applying remedial steps before the release of hatchery fish to improve the survival rate of the fish post-release. This often involves exposure to novel, live food items and conditioning via predator exposure. To date little work of this nature has been done on Australian species. Despite this, large numbers of fish are released into fisheries in Australia each year.

This research examined life skills training, specifically predator recognition, in two species of fish from the troubled Murray-Darling river system, golden Perch (*Macquaria ambigua*) and trout cod (*Maccullochella macquariensis*) and one species, Australian bass (*Macquaria novemaculeata*), from south-eastern Australian coastal river systems. The relative roles of visual and chemical cues in learned predator recognition were investigated, followed by a behavioural interaction experiment with a live predator at liberty in semi-natural conditions. A range of graded responses were observed, however, the intensity of response and preferences for different sensory modalities varied for each species and in different tests. In

golden perch (*Macquaria ambigua*) fingerlings trained using a combination of olfactory cues and conspecific extract (CE) reacted the most intensely. Conditioning using only CE elicited a response that was more generalised than that of the fingerlings conditioned with predator scent and CE, but similar in intensity. In the behavioural predator interaction trial with a predator at liberty in semi-natural conditions, conditioning using a live predator (multiple cues) elicited the greatest response. Fewer approaches toward the predator and an increased use of refuge were observed in fingerlings conditioned with a live predator.

When investigating the Australian bass (*Macquaria novemaculeata*) conditioning fingerlings using only scent cues resulted in an increased use of refuge and a tendency towards looser schooling. Fingerlings conditioned using visual cues or multiple cues recovered faster and exhibited a less intense anti-predator response. Schooling responses were more intense when fingerlings were allowed visual contact with a predator than with the scent of a predator across all treatments. In the behavioural interaction trial with a predator at liberty in semi-natural conditions, combined visual and olfactory cues presented the most intense response, followed by conditioning using only visual cues from the predator. Furthermore, a more distinct difference between treatments was seen in measures of activity rather than of refuge use. Results suggest a stronger partiality to visual cues in this species.

Finally, the results of the trout cod (*Maccullochella macquariensis*) demonstrated the use of multiple cues during conditioning increased the range of responses seen in the fingerlings including predator inspection. Results from the behavioural interaction trial with a predator at liberty in semi-natural conditions also show a stronger response to conditioning using a combination of visual and olfactory cues, or olfactory cues alone. The results presented here suggest the use of chemical and visual cues to enhance predator recognition may be successful in modifying the behaviour of hatchery reared fish in all three study species,

however, these appear to be both context specific and species specific. The use of tailored life skills training programs in these species could potentially improve post-release survival of hatchery reared juveniles.



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This is to certify that I, Erin Kydd being a candidate for the degree of Doctor of Philosophy am aware of the policy of the University relating to the retention and use of higher degree theses as contained in the University's Higher Degree Research Thesis Preparation, Submission and Examination Policy.

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I certify that the work in this thesis "**Life skills training in hatchery reared fish**" has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University. This thesis is an original piece of research written by me. Any help or assistance I have received has been appropriately acknowledged.

In addition, I certify that all information sources and literature used are indicated in the thesis.

The research presented in this thesis was approved by the Macquarie University Animal Ethics Committee, AEC reference number: **2009/046**.

A handwritten signature in cursive script, appearing to read "Erin Kydd".

Erin Kydd (40535541)

06/01/2014

Statement of contribution

The proportions outlined below reflect the contributions by myself and others to the work of all chapters.

Problem formulation; the initial formulation of this problem was contributed by Dr Culum Brown and industry partners. Further formulation of the finer details was my own work, under the guidance of Dr Culum Brown.

Experimental Design; The majority of this work was designed by myself and based on research referenced in text, under the guidance of Dr Culum Brown.

Data collection; All data collection was performed by myself with small contributions made by volunteers.

Statistical analysis; The organisation of data and performing of tests was largely done by myself. Significant contributions were made by Dr Culum Brown including advice on appropriateness of tests.

Results interpretation; The initial interpretation of results is my own work with guidance in the possibility of alternative interpretations contributed by Dr Culum Brown.

Paper presentation; The written work presented is my own work with significant contributions during editing from Dr Culum Brown.

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Chapter 1- Literature Review

Behavioural deficits in hatchery reared fish and remedial steps to improve survival

Each year hatcheries raise vast quantities of fish, valued by anglers and conservationists alike. Many of these are raised with the intention of releasing them into the wild to maintain or bolster existing populations that are depleted as a result of habitat degradation, overfishing or a combination of factors. Since European settlement, Australia's freshwater ecosystems have suffered greatly from a combination of habitat destruction, over exploitation and poor management. The Murray-Darling Basin of South Eastern Australia contains three of Australia's longest rivers, the Murrumbidgee, the Murray and the Darling and is of significant importance to the agricultural industry of Australia. It comprises a large catchment that covers more than a million square kilometres yet much of the rivers within are modified by altered hydrology and habitat degradation (Walker and Thoms, 1993; Norris *et al.*, 2001). The fish fauna found therein contains many popular angling species many of which are now popular eating fish and are commonly commercially stocked. Unfortunately, a number of these species are now listed as vulnerable, threatened, endangered or critically endangered.

Australia has a history of fisheries restocking and the culture of native fish has been taking place in NSW since the 1980's (Rowland, 1983). Improvements in captive breeding techniques have made more species available for restocking into waterways and farm dams. For example, in 2013 the Department of Environment and Primary Industries was involved in the release of over 2.2 million native fish as part of the yearly native fish releases (DEPI, 2013). Whilst there have been examples of successful restocking of Australian native fish, such as the stocking of trout cod *Maccullochella macquariensis* (Cuvier 1829) leading to self-sustaining populations (Koehn *et al.*, 2013), and many

fish are reared for stocking into impoundments or private farm dams, (Rowland, 1983) survival statistics are still lacking for most of the stocked species in Australia.

Data collected internationally suggest that despite substantial releases, a very large proportion of the fish do not survive in their new environment (Brown and Laland, 2001; Brown and Day, 2002b). This post release mortality can make restocking efforts ineffectual. Large scale restocking of Atlantic cod *Gadus morhua* (L. 1758) on the Norwegian coast, for instance, has not yielded significant changes in the catches (Svasand et al 2000). Likewise, recent data from the UK suggests stocking has made no long term improvement in population numbers (Young, 2013). The mortality experienced by hatchery fish often occurs only a short time after their release (Olla *et al.*, 1998; Brown and Laland, 2001; Brown and Day, 2002b). For example, in a release of white sturgeon *Acipenser transmontanus* (Richardson 1836) in Idaho, average 1st year survival rate of released hatchery reared fish was much lower than the survival rate in following years (Ireland *et al.*, 2002). Likewise, Berejikian *et al.* (1999) found that mortality between groups of hatchery fish that differed in experience was occurring within a week of release.

The literature concerning behaviour and survival of hatchery fish has been largely directed at European and American fisheries, with research on Australian species in its infancy. Restocking in Australia of native freshwater species has been used in the past and continues to be used as a tool for conservation programs and fisheries replenishment. The recovery plan for several species of Australian native fish, including species such as trout cod *Maccullochella maquariensis* involve a captive rearing or breeding component. In a study by Ebner *et al.* (2007), releases of Radio-tracked *M. maquariensis* across three locations in the Murray-Darling found mortality ranged from 61% to 100%. There is limited data available on the recorded survival rates of other restocking efforts in Australia despite the vast amounts of money spent to this end.

A range of reasons account for the high post release mortality observed. Predation and starvation appear to be two of the most significant factors in the survival of hatchery fish in the wild. There are many facets within these two areas where hatchery fish show a deviation in behaviour from wild fish (Brown and Day, 2002b). These behavioural differences may contribute to the poor success of released hatchery fish. The differences in behavioural patterns observed in hatchery fish, as opposed to their wild counterparts, have been an area of concern for over 40 years. Research has now turned its attention to finding possible remedial actions that would result in better post release survival whilst still being economically viable within fisheries enterprises.

1.1 Behavioural deficits

Rearing environment and selection within hatchery environments

There are many ways in which environment shapes an individual's behaviour, which in turn, influences survival. The experience gained within an environment, especially during ontogeny, can have a substantial impact on the behavioural repertoire of fish (Brown, 2006). There is a vast body of literature examining how experience influences aspects of foraging (Warburton, 2003) and anti-predator behaviour (Kelley and Magurran, 2003) in fish. Experience can also affect behaviour in areas such as mate choice and schooling preferences (Spence and Smith, 2007; Kozak and Boughman, 2008). By learning through experience, an individual's behaviour can be finely tuned to the current conditions within the environment. Subsequently, a range of behaviours can be generated from experience within a given environment. Brown and Warburton (1997), for instance, found that both habitat complexity and experience with predators may shape the behaviour of the rainbowfish *Melanotaenia eachamensis* (Allen & Cross 1982). The effect that environmental

experience has on behaviour is particularly notable when comparing the behaviours of wild fish with those reared in captivity.

When one considers the standard environment in which hatchery fish are reared, the contrasts to a wild habitat are striking. In an effort to maximise productivity the fish are removed from many of the selective forces they would have encountered in the wild. In most instances, food is highly nutritious and plentiful and there is little to no threat of predation. In addition to this, the housing itself is bare and devoid of structural complexity. These factors, combined with unnaturally high stocking densities, create a habitat for the fish that is far removed from the wild habitat that many are intended to be released into. Such an environment is unlikely to provide the circumstances that allow for a full expression of behaviour and quickly erodes natural behavioural patterns (Olla *et al.*, 1998). The reasons for this are twofold, firstly, through altered selection regimes within the hatchery environment (Johnsson *et al.*, 1996; Sundstrom *et al.*, 2004) and secondly through a lack of experience that is gained from living within a natural environment (Brown *et al.*, 2011). Both of these factors play a part in shaping the behaviour of an individual. Evidence suggests that many of these changes occur relatively rapidly, in a single generation in some instances (Kostow, 2004; Porta *et al.*, 2007). With the current understanding of a fish's capacity for learning (Coble *et al.*, 1985; Brown and Laland, 2001; Brown and Laland, 2003) it is not surprising that hatchery reared fish are ill equipped with the skills to survive in the wild.

One of the key concerns of restocking programs is the genetic divergence between wild populations and captive ones. As with behavioural discrepancies, the root of this problem lies in the differences between the two environments and the selective forces therein. The hatchery environment provides a different set of selection pressures to those experienced in the wild. Kostow (2004) found that physical differences from the wild phenotype occur rapidly in hatchery fish and are correlated with a decrease in survival, providing a potential mechanism for selection. Changes such as this are

particularly pertinent when the hatchery population has been reared in captivity for multiple generations.

Loss of genetic variation can occur within a single generation of captive breeding, depending on brood stock used (Porta *et al.*, 2007). Loss of genetic variation has been recorded across a number of cultured fish species including Indian major carp *Catla catla* (Hamilton 1822) (Hansen *et al.*, 2006), tambaqui *Colossoma macropomum* (Cuvier 1816) (Calcagnotto and Toledo-Filho, 2000), Senegalese sole *Solea senegalensis* (Kaup 1858) (Porta *et al.*, 2007) to name a few. In some instances this is due to poor management practices or insufficient brood stock numbers (Calcagnotto and Toledo-Filho, 2000; Yokota *et al.*, 2003). The potential consequence of this is a lowered effective population that may lead to an inbreeding depression (Hansen *et al.*, 2006; Porta *et al.*, 2007). This raises concern about the ecological ramifications of released or escaped hatchery fish on wild populations through long term genetic changes. Indeed, changes to fitness and reduced reproductive success of a population have been noted in the offspring of hatchery reared individuals released into natural environments (Araki *et al.*, 2007).

Altering population genetic structure is not the only means through which released hatchery fish may impact on existing wild communities. Behavioural differences expressed by hatchery reared fish may result in competition for resources between the two populations. For instance, McGinnity *et al.* (1997) found that the offspring of hatchery Atlantic salmon *Salmo salar* (L. 1758) compete with and displace the smaller wild salmon. Conversely, Berejikian *et al.* (2001) found wild steelhead *Oncorhynchus mykiss* (Walbaum 1792) more socially dominant to hatchery fry reared in impoverished ponds, highlighting the variable and unpredictable ways in which hatchery environments can alter behavioural patterns. Many of these differences are mediated through the high energy demands (and expectations) of hatchery fish. They often have high metabolic rates, fast growth and have poor conversion ratios. This increased demand for food can have flow on effects

such as an increase in the tendency to take risks in foraging contexts or poor performance in the wild (Huntingford, 2004; Saikkonen *et al.*, 2011).

Finding appropriate food and avoiding becoming a meal for another are of critical importance in survival and are the two most significant skills that hatchery fish fail to express after release (Olla *et al.*, 1998; Brown and Laland, 2001; Brown and Day, 2002a). Much of the predation of released hatchery fish generally occurs shortly after their release (Olla *et al.*, 1998; Brown and Laland, 2001; Brown and Day, 2002b) and is often credited to the underdeveloped behavioural proficiency and life skills of the hatchery fish.

1.1.1 Behavioural deficits in predator recognition, response to predation threat and anti-predator behaviour

Pressure from predation is, undoubtedly, one of the most critical issues faced by hatchery fish post release. Predation is unique in its learning parameters in that it generally has little allowance for mistakes. When learning other life skills, such as foraging, the cost of learning from experience is low and the future will often offer another opportunity to learn these skills. Learning about predators, however, rarely affords a second chance and mistakes are costly. Typical responses of fish under threat of predation may include behaviours such as freezing, dashing and tighter shoal cohesion, use of cover, and other locomotor responses (Seghers, 1974; Smith, 1992; Krause *et al.*, 1998). The responses of fish to predation threat have been extensively studied and are often employed when assessing the response of hatchery and wild populations to predation stimuli (for example see Álvarez and Nicieza, 2003; Berejikian, 1995).

Evidence gathered from multiple studies suggest that anti-predator behaviour is highly sensitive to domestication (Berejikian, 1995; Johnsson *et al.*, 1996; Johnsson *et al.*, 2001). Alvarez and Nicieza (2003) found that hatchery rearing of first and second generation wild brown trout *Salmo trutta* (L. 1758) offspring made them insensate to predation risk. They suggested that this behavioural deviation was a result of both selective forces within the rearing environment and differential experience with predators. However, this may have occurred due to differences in origin between the populations examined. In a similar study, Berejikian (1995) also found the ability of fry to avoid predation was negatively affected by both hatchery rearing and lack of experience. The hatchery environment allows few, if any, opportunities for learning about predation and hatchery practices themselves may alter anti-predator behaviour. The use of growth hormones, for instance, has been shown to affect anti predator responses in fish through altered resource allocation due to higher energy demands and feeding motivation (Johnsson *et al.*, 1996). There are a number of aspects in avoiding predation where hatchery reared fish fail that deserve consideration when reviewing the effects of hatchery rearing. These include recognition of predators and response under threat of predation, as well as behavioural divergences that make hatchery fish more vulnerable to predation.

A fish must first be able to correctly identify a predator before it can produce an appropriate anti-predator response. Although differences in anti-predator behaviour between populations may stem from a genetic basis (Magurran, 1990; Huizinga *et al.*, 2009), predator recognition appears to have a largely learnt component (Kelley and Magurran, 2007). When exposed to a novel predator, the hatchery reared offspring of wild fish have been found to show inappropriate or altered anti-predator responses (Magurran, 1990). This implies that there is an association between predators and the cues they emit that must be modified through experience. Furthermore, predator recognition appears to develop relatively rapidly. For instance, predator naïve fathead minnows *Pimephales promelas* (Rafinesque 1820) show a significant fright response to chemical cues of newly

introduced predator fish after only four days of cohabitation (Brown *et al.*, 1997). Likewise, Brown and Smith (1998) showed an increase in anti-predator behaviour after only one experience with chemical cues from a predator.

The use of chemical cues in predator recognition allows fish to gain information at a relatively low risk to their survival. Chemical, or olfactory, cues can spread rapidly throughout the aqueous environment and, without being in close proximity to a predator, can reveal useful information about predation risk (Wisenden, 2000). They may also provide details on aspects of the predator such as diet (Chivers and Mirza, 2001). It has been suggested that they function as a warning that a predator is nearby (Chivers *et al.*, 2001). It appears that many fish have a strongly innate response to some chemical cues, such as conspecific alarm pheromones (Brown and Smith, 1997; Brown and Chivers, 2007) and exposure to such chemical cues is all that is required to elicit an anti-predation response (Berejikian *et al.*, 2003; Huizinga *et al.*, 2009). In other instances, a single exposure with a conspecific alarm pheromone paired with a predator cue is sufficient to induce a distinct anti predator response (Brown and Smith, 1998; Berejikian *et al.*, 2003). Whilst hatchery fish may not initially respond to the chemical cues of a predator, the evidence consistently shows the ease with which they can acquire the recognition skills through chemical cues.

Recognition of a predator through visual means is another important mechanism for predator recognition and may be used in conjunction with chemical cues. Visual cues are often temporally more reliable and give accurate information on aspects such as predator size (Chivers *et al.*, 2001) and hunger (Licht, 1989), all of which are important in assessing the potential risk posed by the predator. There is evidence suggesting that visual cues are relied on most during risky behaviours such as predator inspection (Smith and Belk, 2001). Wisenden and Harter (2001) investigated the role of visual cues in conjunction with chemical cues in *P. promelas* and found that in response to

predators, movement was the visual cue of most significance. Similar observations were made by Brown and Warburton (1997) studying the response of rainbowfish *M. eachamensis* to various model predators. Thus, visual information is also important in the assessment of a potential threat.

The threat sensitive hypothesis, coined by Helfman (1989), predicts that the response of prey will match the intensity of the predation threat. In doing so, they avoid unnecessary expenditures of energy or time lost to anti-predator behaviours. Helfman (1989) showed that increasing the proximity of a predator model to damselfish *Stegastes planifrons* (Cuvier 1830) had a corresponding increase in avoidance behaviours. Many of the responses of prey fish to the threat of predation adhere to a threat sensitive pattern and may reflect the threat levels they have experienced during prior encounters with predator cues or the internal state. For instance, *P. promelas* experienced with differing levels of threat retain the same intensity of response to the threat in subsequent trials (Ferrari *et al.*, 2005). Moreover, prey may make use of the variety of cues available to them to assess the risk. Indeed, exposing coral reef fish to only a visual or chemical cue caused a response that was less intense than when visual and chemical cues were presented together (McCormick and Manassa, 2008). Likewise, the addition of visual information to chemical information dramatically alters the behaviour of anemone fish *Amphiprion percula* (Lacepède 1802) (Manassa *et al.*, 2013). By making use of a range of cues the prey fish are also able to compensate for absent or unreliable information from one sensory modality with increased use of the others. For instance a greater reliance on olfactory cues has been noted in unclear water (Hartman and Abrahams, 2000; Webster *et al.*, 2007) or in darkness (Leduc *et al.*, 2010). If the development of predator recognition requires experience with stimuli, then the predator free environment of a hatchery is unlikely to enhance the predator recognition skills of the fish, or their proficiency at interpreting threat levels and predator cues.

1.1.2 Behavioural divergences that increase the risk of predation

Many hatchery fish display limited anti predator skills, placing them at greater risk of predation after release. This may involve altered schooling behaviour, refuge use or failure to exhibit cryptic colouring to name a few examples. For instance, Alvarez & Nicieza (2003) demonstrated that whilst wild brown trout *S. trutta* will increase nocturnal activity in response to predation threat, hatchery reared trout will not. Similarly, rainbowfish *Melanotaenia duboulayi* (Castelnau 1878) reared in captivity for multiple generations show altered schooling choices by failing to prefer familiar individuals (Kydd and Brown, 2009). Altered schooling behaviours such as this may leave them more vulnerable to predation, and could also potentially impede social learning. Schooling and tighter shoal cohesion are well known as an adaptive anti-predator response (for example see Seghers, 1974). However, in the absence of predation, schooling may cease to be adaptive and behaviours such as these may be distorted.

One of the well documented examples of altered behavioural patterns in hatchery fish are those of cultivated flat fish species. Hatchery summer flounder *Paralichthys dentatus* (L.1766) take longer to become cryptic on the benthos than wild summer flounder (Kellison *et al.*, 2000). They suggested that the inability to become cryptic was a result of a lack of experience with natural substrates. Hatchery flounder have also been found to spend significantly more time swimming in the water column, instead of on the benthos, as wild flounder do (Furuta, 1996; Kellison *et al.*, 2000). This behaviour makes the fish more vulnerable to predation. In this instance, the inappropriate behavioural patterns observed in hatchery fish may have been inadvertently encouraged by hatchery practices. For instance, if feed is dispersed across the surface of the water, the hatchery fish are conditioned to forage at the surface. This, in turn, may make the hatchery fish more vulnerable to predation after release, particularly to avian predators.

1.1.3 Deficits in foraging skills

The ability to forage successfully is an essential skill that may potentially determine the survival of an individual. The fish that avoid immediate predation may later suffer from starvation as foraging in wild habitats is vastly different from the feeding routine of hatcheries. The diets of hatchery reared fish vary from the diet of wild fish in many aspects such as food type, variability and abundance. This on its own may present a challenge to the naïve hatchery fish without considering the difficulties of catching and consuming live prey.

The range of food items consumed by released hatchery fish may be disproportionate to those of wild fish, and microhabitats underutilized (Olla *et al.*, 1998). The benthic prey component might be neglected in the diet, possibly due to the fish being conditioned to forage at the water's surface rather than lower in the water column (Ersbak and Haase, 1983). Hatchery fish that have only ever had food delivered at the surface are likely to maintain a strong bias towards this behaviour after release. Reinhardt *et al.* (2001), for example, found hatchery reared masu salmon *Oncorhynchus masou masou* (Brevoort 1856) favour foraging at the surface rather than lower in the water column. This could leave the hatchery reared fish at a disadvantage when foraging in a wild habitat as it limits the area available for foraging.

It is recognised that hatchery fish have trouble distinguishing and handling novel food items (Brown and Laland, 2001) and the stomach contents may be lower than that found in wild fish (Ersbak and Haase, 1983; Munakata *et al.*, 2000). In some cases, hatchery fish have been found to consume small stones and debris that resemble pellets (Munakata *et al.*, 2000; Ellis *et al.*, 2002). Naïve, hatchery fish show a preference for pellet food or may, in fact, attack stones preferentially to unfamiliar live food (Ellis *et al.*, 2002). These behaviours are likely to be a consequence of rearing in the hatchery

environment as they have had little experience with novel food items and develop inappropriate foraging search images.

Foraging skills, in some instances, may take longer to acquire than anti-predator skills and several exposures with a novel prey item may be required. For example, Warburton & Thomson (2006) found that silver perch *Bidyanus bidyanus* (Mitchell 1838) feeding on a novel prey item required five exposures before they were able to forage at maximum efficiency on that prey item. Likewise, foraging on a novel item took hatchery reared turbot *Scophthalmus maximus* (L. 1758) nine exposures to reach the feeding rate of wild turbot (Ellis *et al.*, 2002). In comparison, some anti-predator skills can be acquired after only a single exposure (Brown and Smith, 1998). When released into wild environments after being reared in a hatchery, all prey items are likely to be unknown to the fish, and their foraging experiences may be limited to commercial food. The preference for pellet food over live food may persist for several weeks (Ellis *et al.*, 2002), and although this will diminish with time, it presents another example of the ways in which hatchery rearing impairs behavioural development.

The learning of foraging skills is complicated by many issues and the ability of the fish to adapt will often vary across species and habitat (for review see Warburton, 2003). Despite the shortcomings of learnt foraging skills presented by many hatchery reared fish, the strong influence that experience plays in learning means remedial steps can be taken to overcome many foraging deficits.

1.2 Remedial steps to improve survival

The behavioural repertoire expressed by an animal is a combination of innate responses and responses learned through experience. By understanding and manipulating these aspects it is possible to mediate some of the behavioural deficits observed in hatchery reared fish. A substantial improvement in post release survival of hatchery reared fish could potentially be achieved with a combination of pre-release training and preparation in conjunction with careful planning, appropriate selection of broodstock and a thoughtful release strategy.

1.2.1 Release strategies

When released into the wild, fish undergo an array of physical, endocrinological and behavioural changes in a short amount of time. They do, however, appear to be able to adapt to this (Munakata *et al.* 2000). Nonetheless, their chances of survival may be moderated by poor release practices and stress associated with transport and release. For example, upon release, fish may be suffering from exhaustion making them highly susceptible to predation. Resting the fish prior to release as part of a soft release protocol could allow the fish enough recovery to be able to escape predation. A minimum time of 90 min rest has been suggested by Olla & Davis (1989) to allow stress levels to reduce. Releasing the fish into a predator free enclosure can provide the fish with an opportunity to recover and acclimatise whilst remaining safe from predation, the use of such enclosures has been demonstrated to improve survival rates (Brennan *et al.*, 2006) and is another tool that can be used in optimising the success of stocking efforts. Time spent recovering from stress can also reduce osmotic shock as the fish acclimatize to the water chemistry at the release site. The immediate area into which the fish are released may also improve or reduce the chance of avoiding predation shortly after release. Rooker *et al.* (1998) found this to be the case in red drum *Sciaenops ocellatus* (L. 1766), where release into an un-vegetated area significantly increased chance of predation. In a

similar study by Stunz & Minello (2001), survival after release of wild red drum was increased in complex habitats. However, in the same study, released hatchery red drum showed no improvement of survival in complex habitats and their mortality rates were overall significantly higher than those of the released wild fish. Stunz & Minello (2001) suggest that this is a result of behavioural modification in the hatchery habitat, again highlighting the need for pre-release behavioural preparation. Size at release can also influence survival of stocked fingerlings. Studies of Australian native species, including Australian bass *Macquaria novemaculeata* (Steindachner 1866) and golden perch *Macquaria ambigua* (Richardson 1845), found that the release of fingerlings at 50-65mm usually resulted in greater survival rates than those stocked at smaller size classes (35-45mm and 20-30mm) (Hutchison *et al.*, 2006). However, rearing fish to greater sizes costs more money and prolongs the time in captivity during which behaviour may deteriorate further.

The evidence suggests that to improve survival of released hatchery fish, both the preparation and execution of release must be carefully planned. In addition to reducing stress at release and soft release practices, prior remedial steps that can be undertaken at the hatchery that may also improve the survival of hatchery fish post release. The application of behavioural conditioning to hatchery reared fish, with the view of improving survival in a wild environment, is referred to as life skills training.

1.2.2 Life skills training

We now realise that learning plays a key role in the development of fish behaviour in virtually all contexts (Brown *et al.*, 2011). Our improved understanding of the ability of fish to learn means that many behavioural deficits are in some way reversible using carefully planned remedial methods. Life skills training refers to the pre-release training of fish to recognise food types and predator threats

(Brown and Laland, 2001). The use of life skills training has been well researched for salmonid species, however research on Australian native and other cultured species is only in its infancy.

1.2.3 Anti-predator skills

Whilst some anti-predator skills are innate, experience is required to hone these skills and act as a catalyst for learning. Life skills training for avoiding predation in hatchery fish has focused on enhancing predator recognition skills as well as anti-predator behaviours, often through controlled experiences with predators or predator cues. The aim is to provide some of the experience with predators, in a non-lethal situation, that the hatchery environment does not typically offer. This may involve the use of stimuli (such as a model predator or chemical cues) that will elicit an anti-predator response and facilitate learning whilst providing minimal damage and mortality to the fish.

The use of predator models for reducing vulnerability to predation has shown some promising results. Including the modelled impact of predation and enrichment through live feed, for example, enhanced the feeding and defensive behaviours of hatchery bream *Abramis brama* (L. 1758) demonstrating the role of environmental information during rearing (Gerasimov and Stolbunov, 2007). Hossain *et al* (2002), found hatchery Japanese flounder *Paralichthys olivaceus* (Temminck & Schlegel, 1846) learned quickly about predation by crabs. By using small, benign predator crabs or by fencing off larger ones, they were able to condition the hatchery flounder to crab predators, and reduce their chance of predation. Similarly, anti-predator conditioning of hatchery Summer flounder *Paralichthys dentatus* (L. 1766) made them significantly less prone to predation by crabs than naïve flounder (Kellison *et al.*, 2000). However, Kellison *et al.* (2000) found wild flounder were still better able to avoid predation than the conditioned hatchery flounder. It appears that whilst the behavioural deficits can be reduced, they are difficult to correct completely.

Another tactic that is commonly employed in training for predator avoidance is the use of chemical cues. Chemical cues allow for information to be gathered from a distance, providing a low risk form of information transfer (Kats and Dill, 1998). Chemical cues, when paired with alarm cues such as alarm pheromones from conspecifics, aid in the acquisition of predator recognition and many fish species possess a strongly innate reaction to conspecific alarm pheromones (Brown, 2003). By mimicking the odour of a damaged conspecific, there is a unique opportunity to condition an aversive response in hatchery fish by pairing it with novel stimuli, such as a predator cue. Berejikian *et al* (1999) found that the use of acquired predator recognition (through association between predator odour and conspecific alarm cues) showed an improvement in survival after less than a week post release. Conditioning using chemical cues appears to take relatively few exposures to generate an appropriate response. In an experiment where juvenile Chinook salmon *Oncorhynchus tshawytscha* (Walbaum 1792) were treated with predatory pikeminnow *Ptychocheilus oregonensis* (Richardson 1836) odour along with conspecific extract, only one exposure was needed to elicit enhanced predator recognition (Berejikian *et al.*, 2003). Recent studies have shown that fish are more sensitive to learning these skills during specific stages of their ontogeny (Hawkins *et al.*, 2008). Likewise, it has been demonstrated that learned predator recognition can start before hatching and learned associations can begin during embryonic stages (Nelson *et al.*, 2013; Oulton *et al.*, 2013). Recognising these stages has the potential to improve life skills training attempts.

1.2.4 Foraging skills

Training hatchery fish to recognise novel food items is relatively simple in its execution, and usually involves feeding a range of food items to the naïve fish prior to liberation. Multiple exposures are generally required to reach maximum foraging rate. Turbot *Scophthalmus maximus* (L. 1758), fed on live feed reached the foraging efficiency of wild fish after nine days of exposure to live food (Ellis *et al.*, 2002). Likewise, Warburton & Thomson (2006) found that silver perch *Bidyanus bidyanus* (Mitchell 1838) required 5 exposures before they were able to forage on a novel food item at

maximum efficiency. As such, training naïve fish to recognise a new food item is as simple as allowing them repeated opportunities of feeding on the novel prey item. There are, however, other means of improving the foraging skills of hatchery fish.

In some instances, the location that food is dispersed can improve the foraging abilities of the hatchery fish. Salmon, for instance, can be easily conditioned to forage on the benthos, rather than on the surface. Brown et al (2003b) successfully conditioned hatchery reared *S. salar* to forage near the benthos after only six days of exposures where they observed others foraging. By feeding the fish below the water surface, they are encouraged to forage at different depths, thus promoting more natural foraging behaviour. Feeding on the benthos has the added benefit of encouraging fish to swim in a more appropriate area within the water column rather than near the surface, which may have beneficial follow on effects, such as reduced exposure to avian predators.

Foraging skills, like many other behavioural patterns, can quickly be acquired through social learning. Observation of con-specifics feeding is a strong social facilitator, making social cues a potential mechanism to exploit for the purposes of life skills training (Suboski and Templeton, 1989). Brown *et al.* (2003b) found that observation alone was sufficient to teach *S. salar* to forage for benthic prey items. Likewise, observation of conspecifics feeding on live prey items increases the foraging success of naïve *S. salar* (Brown and Laland, 2002). In terms of life skills training, this implies that not all the fish need undergo intensive foraging training, only some individuals that can then serve as demonstrators when reintroduced to the remaining, naïve fish.

Many fish species have a great capacity for social learning (Brown and Laland, 2003), this is a tool that can be readily employed in training life skills to fish for release. The schooling behaviour of many species (particularly as juveniles) may also be of utility in training through social facilitation and social learning. It may be possible to use social cues to enhance the learning and recognition of a

novel stimulus, for instance. Indeed, this tactic has been used with success in the training of foraging skills and predator recognition in fish intended for release (for review see Suboski and Templeton 1989; Brown and Laland, 2001).

1.2.5 Environmental enrichment

Environmental enrichment generally refers to the addition of physical components within the hatchery environment that increase the spatial complexity. However, it can include any aspect of husbandry that increases heterogeneity of the environment, encouraging natural behaviour in the animals or improving biological functioning (Newberry, 1995). This includes enhancement of the environment using such things as overhead or submerged cover (for example see Berejikian *et al.*, 2001) or may be as simple as providing water movement (Gerasimov and Stolbunov, 2007) or feeding in different locations. The aim of environmental enrichment is to provide aspects of the hatchery environment that are akin to those in the wild, thus facilitating the expression of more naturalistic behaviours.

There is evidence to show that providing an enriched habitat during ontogeny may stimulate the growth and development of the brain (Kihlslinger and Nevitt, 2006). By simply enriching the environment (through spatial complexity) that fish are raised in, the ability of the fish to learn foraging tasks can be improved (Brown *et al.*, 2003a). Rearing in enriched environments has been shown to improve neural plasticity and cognition and improved learning in spatial tasks (Salvanes *et al.*, 2013). Cod reared in enriched, variable environments differ from standard hatchery reared fish in many aspects including refuge use, foraging skills, activity levels and behavioural flexibility (Braithwaite and Salvanes, 2005). These are all skills that could contribute to the success of the fish in the wild. Berejikian *et al.* (2001) found that environmental enrichment resulted in more socially dominant fish than standard hatchery rearing. However, they still differed from wild populations in

the amount of territory overlap, thus, environmental enrichment in this instance can improve some behavioural differences, but not all. Environmental enrichment has the potential to provide some improvements to the behavioural repertoire of hatchery fish with a minimal amount of effort and could easily be implemented into many existing hatchery facilities.

1.3 Conclusion

Significant improvements in post-release survival could be made by amending the ways in which hatchery fish are reared, prepared for release and finally released. The increasing body of literature on life skills training suggests that the primary problems of hatchery fish failing to respond appropriately to predators and live prey are, at least partially, reversible. These remedial steps need not be costly and have the potential to dramatically increase post release survival of hatchery fish.

Restocking, however, should not be considered in place of sustainable management or to avoid addressing more serious problems. It need hardly be mentioned that these methods would be best applied in conjunction with habitat restoration and maintenance, as restocking of fish into an area that is uninhabitable for them would be futile. The use of hatchery reared fish for restocking is not without its problems and it may indeed damage natural populations if done carelessly. However, thoughtful planning and thorough research should avoid such problems.

Whilst there is a large body of work on life-skills training concerning Salmonid species, little has been done pertaining to Australian species. With the Murray-Darling basin being a freshwater

habitat suffering from several threatening processes, there is a great need for further study on the ecosystems and the biota within. As hatcheries compromise normal behavioural patterns, including, but not limited to, foraging success and predator recognition and avoidance, a more thorough understanding of these deficits should be investigated in species that are used in restocking.

In this thesis, the behaviours of three species of stocked Australian native fish will be examined, golden perch *Macquaria ambigua* (Richardson 1845), Australian bass *Macquaria novemaculeata* (Steindachner 1866), and trout cod *Maccullochella macquariensis* (Cuvier 1829). Specifically, predator recognition (through visual and chemosensory mechanisms) and the relative roles of the different sensory modalities will be assessed in the context of life skills training. Following this, the value of the life skills training is examined in a behavioural interaction trial, where fingerlings with varying degrees of predator conditioning are exposed to live predators at liberty in a semi-natural setting. Through these experiments it is hoped that a better understanding of predator conditioning and the relative roles of visual and olfactory cues in these three species of fish will be established.

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Chapter 2

Predator conditioning in hatchery reared golden perch *Macquaria ambigua*:

The roles of visual and olfactory cues in learned predator recognition

This chapter is formatted for publication in the Journal of Fish Biology. All figures and tables are embedded in text.

2.1 Abstract

For juvenile fish it is vitally important to be able to recognise a predator and assess the risk it poses. For fish reared in hatcheries there is little opportunity to learn about predators which can lead to numerous behavioural deficits. Pre-release training involves conditioning the fish to associate predator cues with aversive stimuli, usually the scent of a damaged conspecific. Here we investigate the roles of different predator cues in learned predator recognition in golden perch *Macquaria ambigua*, a commonly stocked and popular angling fish from the Murray-Darling region of Australia. The results indicate that juvenile golden perch were able to recognise a predator when conditioned using predator cues combined with conspecific extract. Conditioning using only the olfactory cues from the predator combined with conspecific extract resulted in a more dynamic range of anti-predator responses. A graded response was shown by fish conditioned using visual cues or a combination of cues in what is likely a threat sensitive response. These results demonstrate the potential for life skills training to be utilised in this species prior to release, which may improve their ability to avoid predation post-release.

Key words: *Macquaria ambigua*, learned predator recognition, anti-predator conditioning, hatchery reared, olfactory cues, visual cues

2.2 Introduction

Correctly recognising and assessing the risk posed by a predator is one of the challenges faced in the lives of prey animals. In fish, predator recognition and the nature of the anti-predator response are often fine-tuned to suit local conditions. This develops as a result of learning predator specific information which allows the individual to tailor their responses to contemporary environmental variables (Kelley and Magurran, 2003; Griffin, 2004). A variety of cues are available for prey to do this, including visual and olfactory cues transmitted by the predator. How they use these cues, both together and in isolation, is likely to vary from species to species and between different habitats. For example, guppies *Poecilia reticulata* (Peters 1859) reared in low light conditions compensate for reduced visual clarity with an increase in use of olfactory cues, demonstrating the plasticity of sensory modalities (Chapman *et al.*, 2010). In addition, prior experience with predators is also liable to change the type and intensity of the response offered (Magurran, 1990).

The threat sensitive hypothesis predicts that prey will match the intensity of their response to predator cues to the perceived threat level (Helfman, 1989). The level of risk is partially determined by environmental conditions (eg availability of cover), the nature of the predator (eg size or species), the size of the prey and its internal state (eg hunger levels). As part of this equation, therefore, prey must be able to recognise and make an accurate judgement of the threat posed by a predator. Over reacting to a non- threatening predator is time and energy wasted (Lima and Dill, 1990), however, failure to react to a predator that results in capture is very costly indeed. Thus, the cues received and the information, and by extension threat level, they entail are expected to impact on the anti-predator behaviours exhibited by an individual.

The aqueous environment lends itself to extensive use of olfactory cues and fish are well suited to detect chemical cues. Olfactory cues are useful in detection of predators in situations where vision is limited such as in highly structured, dark or turbid waters (Kats and Dill, 1998). However, they do have limitations. Visual cues have the benefit of providing more temporally specific information,

useful for accurately assessing potential risk. Chemical cues, however, likely act as an early warning system. The use of olfactory cues in predator recognition is widespread in fish and conspecific alarm cues are a well-known facilitator of learned predator recognition (Smith, 1992; Kelley and Magurran, 2003; Brown and Chivers, 2007). Predator recognition by naïve fish is achieved through associative learning, whereby an alarm cue released from damaged conspecifics is paired with a predator cue (Suboski *et al.*, 1990). The effectiveness of this system is that the innate response released following detection of alarm substances can be paired with any potential threat in the environment and thus offers a high degree of flexibility.

Predator avoidance using chemical cues has been extensively studied in the context of the threat sensitive hypothesis (Ferrari *et al.*, 2010a) and the relative roles of chemical and visual cues appears to vary widely. For instance, Martin *et al* (2010) showed that juvenile roach *Rutilus rutilus* (L. 1758) reacted to olfactory and visual cues from two types of predators in a complex and variable style with different anti predator behaviours exhibited. In instances where there are multiple cues provided, the multimodal information may have an additive effect (Smith and Belk, 2001). Other studies have noted a graded effect in behaviour when comparing information gathered from different sensory modes. For example, newly settled reef fish respond in a variable manner to visual and olfactory cues dependant on the intensity and modality of the predator cue (Holmes and McCormick, 2011). Further to this, when one sensory modality is compromised, such as in situations of low visibility, then prey may rely more heavily on an alternative source of information (Hartman and Abrahams, 2000). All these factors combine to create a context specific response that improves chances of survival at the lowest possible cost to the individual.

The experience of an individual is another factor that is likely to contribute to the anti-predator response a fish exhibits in the presence of a predator. For example, juvenile coral reef damselfish *Pomacentrus wardi* (Whitley 1927) that had experience with a predator, regardless of whether through visual or olfactory cues, displayed less hazardous behaviours (Lönnstedt *et al.*, 2012). This

type of experience is essential to prey recognising and avoiding predation threat. There is also evidence that recent experience shapes anti-predator response and intensity. For instance, Brown *et al.* (2006) found that prey fish alter their anti-predator behaviour and response threshold according to the background levels of threat they had experienced over the previous three days. Moreover, recent evidence has shown that embryos can not only differentiate between predators (Oulton *et al.*, 2013), but the associative learning process can begin to shape the behaviour of fish even during embryonic development (Nelson *et al.*, 2013). It is details such as this that allow a fine tuned response to local predation threat, while not expending any energy on anti-predator behaviour unnecessarily.

Research in the area of learned predator responses has focused mostly on olfactory cues in species such as guppies *P. reticulata*, minnows *Pimephales promelas* (Rafinesque 1820) and salmonids (Ferrari *et al.*, 2010a; Brown and Chivers, 2007). There are limited studies of this type focusing on Australian native species of freshwater fish. This is surprising given the extensive stocking programs in place in Australia, particularly the Murray-Darling region of NSW and Victoria, which could benefit from an improved understanding of the details of predator recognition in the stocked species. Golden Perch *Macquaria ambigua* (Richardson 1845), are a large freshwater fish species found in slow flowing, lowland areas (Lintermans, 2007). They are a popular target species with anglers and as such have been the subject of large scale stocking programs which see several hundred thousand fingerlings liberated each year. To date there is little information available on the success of such programs, however, global literature suggests survival of stocked fish is usually poor (Olla *et al.*, 1998; Brown and Laland, 2001).

In response to the poor post-release survivorship of hatchery-reared fishes, many have advocated the use of pre-release training (Brown and Laland, 2001). The theory driving life-skills training is that it provides naïve, captive-reared individuals the opportunity to learn skills such as foraging and predator avoidance prior to their release. In a practical sense, this often involves multiple exposures

to predators or live prey in the captive environment prior to release. However, it can be as simple as enriching the rearing environment or feeding live prey (Brown *et al.*, 2003; Czerniawski *et al.*, 2011). Such practices are common place in conservation biology but are seldom applied to fishes (Brown and Day, 2002). Gaining a better understanding of how the different sensory inputs function in learned predator recognition is crucial to allow for a more tailored approach to pre- release conditioning, with the aim of improving post release survival of liberated fish.

In this study we investigated the relative roles of visual and olfactory cues in learned predator recognition in juvenile golden perch *M. ambigua*. We conditioned predator naïve golden perch *M. ambigua* fingerlings using paired predator cues and damaged conspecific odours. The response of the fingerlings to the presence of a live predator was then tested. Two questions were addressed, 1) do the fingerlings achieve learned predator recognition through associative learning with predator cues and 2) what combination of cues elicits the most effective response. The results described here begin to characterise the anti-predator responses peculiar to this species and will help to illustrate the relative roles of different predator cues in learned predator recognition. Moreover, the results enable us to tailor life-skills training for this species with a view to bolstering post-release survival of hatchery-reared individuals.

2.3 Methods

2.3.1 Subjects and Housing

Young of the year Golden perch *M. ambigua* fingerlings, of around 5cm TL, reared in outdoor dams by a commercial supplier under the Hatchery Quality Assurance Scheme (HQAS) were used for this experiment. The fingerlings were shipped to the university overnight in plastic bags, augmented with pure oxygen, inside polystyrene crates. Upon arrival at Macquarie University, the fish were moved to 120 l tanks (measurements 90x30x35cm) in a laboratory. Here they were housed in groups of 100 fingerlings per tank until experimental conditioning commenced. The laboratory was maintained at

19°C and a 12hrs light: 12hrs dark light cycle. The fingerlings were fed daily on frozen bloodworms. They were housed this way for one week to allow them to acclimatize to laboratory conditions and to recover from any stress of travel prior to the commencement of experiments.

Spangled perch *Leiopotherapon unicolor* (Günther, 1859) (n = 3) were collected from the wild for use as predators. The predators were shipped to the university in plastic bags, augmented with pure oxygen, inside polystyrene crates. All were mature, measuring between 15 and 20cm. They were individually housed in the laboratory in 60l tanks heated to 19°C and fed a mixture of earthworms and frozen prawns. They were not fed for the duration of the experiment to encourage more hostile behaviour. During testing, the predator in use was alternated with each day to ensure the predators remained interested and active.

2.3.2 Conditioning

A total of 24 replicate groups were conditioned per treatment. Each of the four treatments was conditioned in their own tank. Fingerlings were conditioned in batches of 25 fingerlings per tank. This constituted 8 replicate groups of 3 fingerlings per conditioning tank, with an additional fish to spare. Three batches were conditioned per treatment to achieve the total of 24 replicate groups, however, some fish in poor condition were not ultimately tested. Each tank was designated to one of the 4 treatments. The fingerlings were moved into their conditioning tanks 1hr prior to the first exposure to allow them to settle sufficiently. Each tank was exposed 3 times per day to their treatment over three days (total of 9 exposures). Each exposure lasted 15min. A series of pilot studies suggested that this was the optimal training regime. This was done at between 9am – 10am, noon – 1pm and 3pm – 4pm. Chemical cues were administered slowly to avoid startling the fish unnecessarily. At the completion of the conditioning, each tank was drained and re-filled with de-chlorinated water.

2.3.3 Treatments

The ability of the fingerlings to learn to recognise predators was investigated using combinations of chemical and visual cues. The following four treatments were used:

2.3.3.1 Conspecific extract

To create conspecific extract (CE), whole carcasses of conspecifics (3000mg) were homogenized. This amount was extrapolated from the weight of conspecific skin used in previous studies (Brown and Smith, 1998). As there is no information available on specific alarm pheromones in the study species, the decision was made to use the whole carcass, rather than just the skin. The 3000mg of homogenised conspecific was then added to 100ml of distilled water and filtered through coarse filter paper (Advantec 6µm). Once this was done, more distilled water was added to bring the solution total up to 400ml. This was then poured into ice-cube trays, each cube containing 20ml of conspecific extract. The ice-cube trays were then frozen until use. The CE treatment received only 20ml of conspecific extract at conditioning times with no associated predator cues.

2.3.3.2 Predator scent plus conspecific extract

During this treatment (henceforth abbreviated to predator scent treatment) fingerlings received a combination of conspecific extract and predator scent only, with visual cues excluded. This is the standard approach to entrain anti-predator behaviour in fishes (Brown *et al.*, 2011). Water that contained the chemical signatures of the predator was used as predator scent. Immediately prior to exposure, 20ml of water was collected from a 60l tank containing a single Spangled perch *L. unicolor* who had been housed in the tank for an extensive period of time. This volume was similar to that used in studies by Brown & Smith (1998) but was reduced in volume to adjust for the extended

housing of the *L. unicolor* in the tank prior to collection. The *L. unicolor* was fed a diet of frozen prawns 4 times per week, thus there should be no dietary influence on the odour. Feeding was suspended during experimental periods. The tank had no water changes and was not cleaned for a week prior to the conditioning stage. At conditioning times, the scent was added directly to the conditioning tank and immediately followed by the addition of 20ml CE to the tank.

2.3.3.3 Live Predator (visual and chemical predator cues) plus conspecific extract

During this treatment (henceforth abbreviated to live predator treatment) a partition made from fine mesh (2mm²) was placed at 1/3 of the length of the tank creating two sections; a large section (62cm x 35cm depth x 35cm wide) and a small section (31cm x 35cm depth x 35cm wide). A *L. unicolor* was placed in the small section of the tank whilst the fingerlings were placed in the large section. Water was able to pass freely through the partition allowing good circulation and facilitating movement of chemical cues between the compartments. An opaque partition placed next to the mesh was attached to a pulley and lifted at each exposure for a duration of 15min to allow visual contact with the predator. This was done in conjunction with the addition of 20ml CE. The predator was removed over night to prevent it attempting to escape.

2.3.3.4 Control

The control fish were not exposed to any cues. They were simply removed from the housing tank and placed in aquaria of the same dimensions as the other treatments for the equivalent amount of time. The control group were representative of regular hatchery fish which have no conditioning prior to release.

2.3.4 Experimental Procedure

After the 3 days conditioning the fish were tested. To do this they were placed in groups of 3 in an experimental tank 92cm long x 51cm deep, filled to a depth of 31cm (Fig. 2.1). The experimental tank consisted of 2 compartments, separated by a clear Perspex partition placed at approximately 1/3 of the length of the tank (30cm). The partition allowed visual contact between both sides and had small holes to allow movement of water and chemical cues. In the Predator zone, gravel was placed on the bottom, primarily to block the bottom and edges of the partition and prevent fingerlings from reaching the predator zone. The 1/3 of the tank that was furthest from the predator was divided into 2 'zones', marked on the underside of the tank. One of these zones was designated the planted zone and was furnished with four plastic plants, evenly spaced within the zone. The other zone was designated the 'open' zone and contained nothing. This was included to distinguish any preferences for the planted zone as being distinct from distance from the predator. The area nearest the predator zone was termed the 'near' zone. Entering the near zone was likely to reflect predator inspection whilst use of the planted zone was likely to indicate refuge use. An opaque barrier was also placed in the tank, next to the Perspex barrier, separating the predator from the fingerlings visually and limiting the transmission of olfactory cues. This was attached to a pulley, and could be lifted remotely to allow visual contact between the fingerlings and predator.

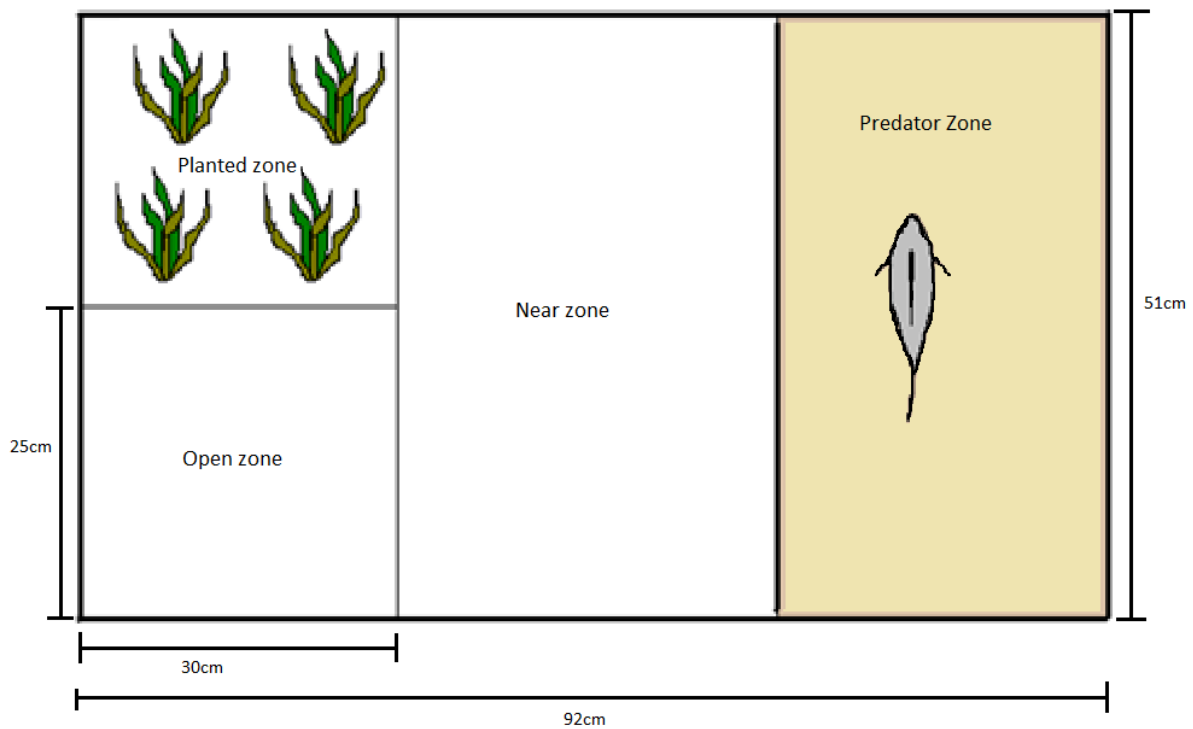


FIG. 2.1 Diagram of experimental tank used for the area use live predator test. Water was maintained at a depth of 31cm. Gravel was placed on the base of the predator zone and the planted zone was furnished with four plastic aquarium plants.

Fingerlings were tested in eight replicate groups of 3 to encourage activity in timid fish that may otherwise remain immobile. They were placed in the near zone of the testing tank and allowed 20 min to settle prior to any recording. Behaviours were recorded on an overhead camera for 20 min before the opaque barrier was lifted, then for a further 20 min after the barrier was lifted, while the predator was visible. The movements of each of the 3 fingerlings were recorded and a group average taken. Behaviours recorded include use of the different zones, termed area use, and number of border crossings, termed activity.

2.3.5 Data Analysis

The average of the three fish was taken as the data point since the fish school. Data for open zone use, near zone use and borders crossed was log transformed to achieve normality. The data for planted zone use was square root transformed. Data was then reduced to the 10min prior to the barrier being lifted and the 10min after the barrier was lifted and analysed using ANOVA and repeated measures ANOVA. The training treatments were used as independent variables and the values before and after exposure to the predator treated as the repeated measures. Data were further scrutinized using a series of post-hoc tests as required. Post-hoc tests used included one-way ANOVA and pairwise comparisons. All data analysis was conducted using Statview 5.0.1 (SAS Institute Inc.).

2.4 Results

The use of the near (Fig. 2.2), open (Fig. 2.3) and planted (Fig. 2.4) zones by conditioned fingerlings was investigated using a repeated measures ANOVA. Predominantly, the fingerlings of all treatments spent the majority of their time in the planted zone, both before the predator was revealed and after the predator was revealed (see Fig. 2.2, 2.3 and 2.4). No main effect of treatment was found in the use of any zone; planted ($F_{3, 78} = 1.18, P = 0.322$), open ($F_{3, 78} = 1.17, P = 0.327$) or near ($F_{3, 78} = 0.95, P = 0.420$), nor was any time effect found for near zone use by conditioned fingerlings ($F_{1, 78} = 3.13, P = 0.081$). A strong effect of time, however, was evident in open zone use ($F_{1, 78} = 14.77, P = 0.0002$) and also in planted zone use ($F_{1, 78} = 4.07, P = 0.047$). Following exposure to the predator, fingerlings tended to increase their time in the near zone and decrease the time in the open and planted zones. There was no treatment by time interaction in the use of the open zone ($F_{3, 78} = 0.63, P = 0.597$), or in the use of planted zone ($F_{3, 78} = 0.35, P = 0.789$). However, there was a treatment by time interaction for the use of near zone by conditioned fingerlings ($F_{3, 78} = 4.07, P = 0.010$). Closer

scrutiny of the data showed that fingerlings from the control treatment did not significantly change in zone use after the predator was revealed in either the planted, open or near zones. However, fingerlings from the predator scent treatment significantly increased their use of the near zone after the predator was revealed ($F_{1,18} = 10.51$, $P = 0.005$) and reduced their use of the planted zone ($F_{1,18} = 6.73$, $P = 0.018$). Pairwise post-hoc comparisons showed an increased use of the near zone by fingerlings in the predator scent treatment after exposure to the predator which was significantly greater than that of the live predator ($F_{1,38} = 9.20$, $P = 0.004$) and conspecific extract treatments ($F_{1,36} = 6.79$, $P = 0.013$). Neither the live predator nor the conspecific extract treatments were statistically different from the control treatment ($P > 0.5$ in both cases).

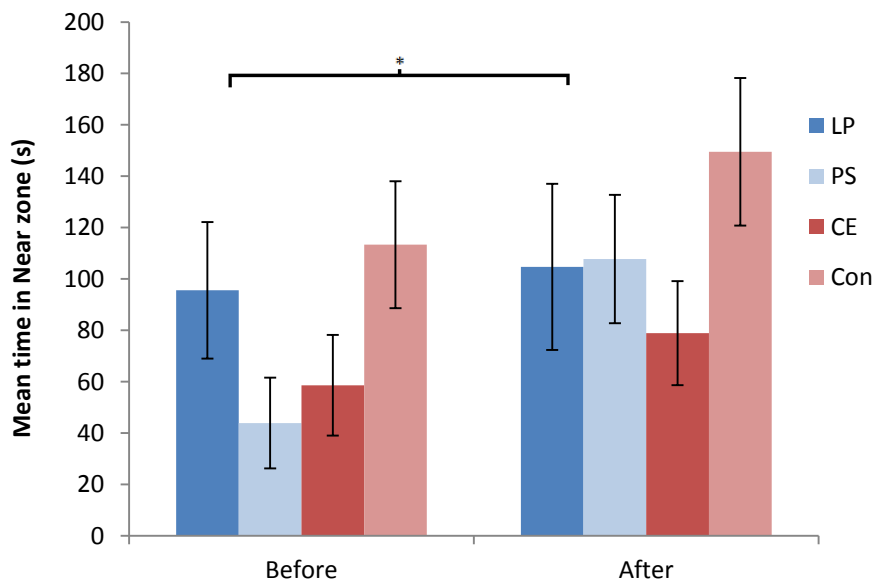


FIG. 2.2 Mean (\pm SE) time spent in the near zone by conditioned fingerlings in the 10 minutes prior to the predator being revealed and the 10 min after the predator was revealed. Fingerlings were conditioned with one of four treatment; live predator (LP), predator scent (PS), conspecific extract (CE) and control (Con). (*indicates $P < 0.05$).

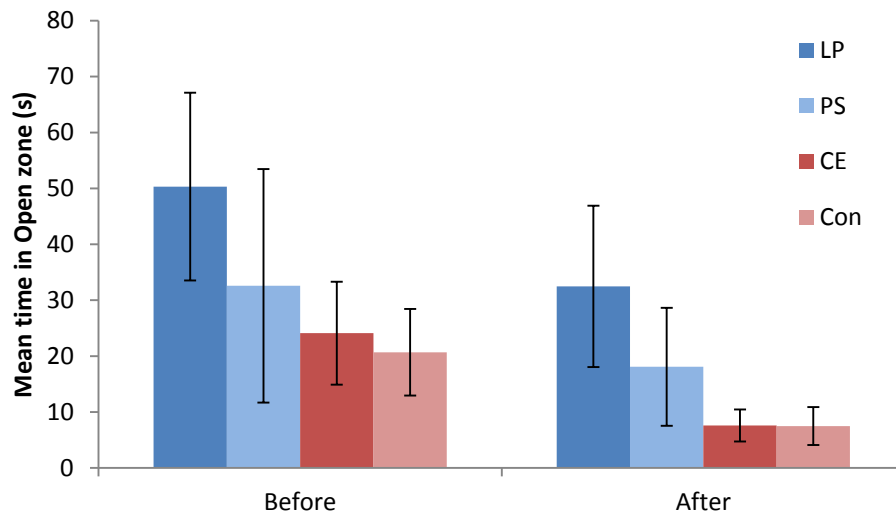


FIG. 2.3 Mean (\pm SE) time spent in the open zone by conditioned fingerlings in the 10 min prior to the predator being revealed and the 10 min after the predator was revealed. Fingerlings were conditioned with one of four treatment; live predator (LP), predator scent (PS), conspecific extract (CE) and control (Con).

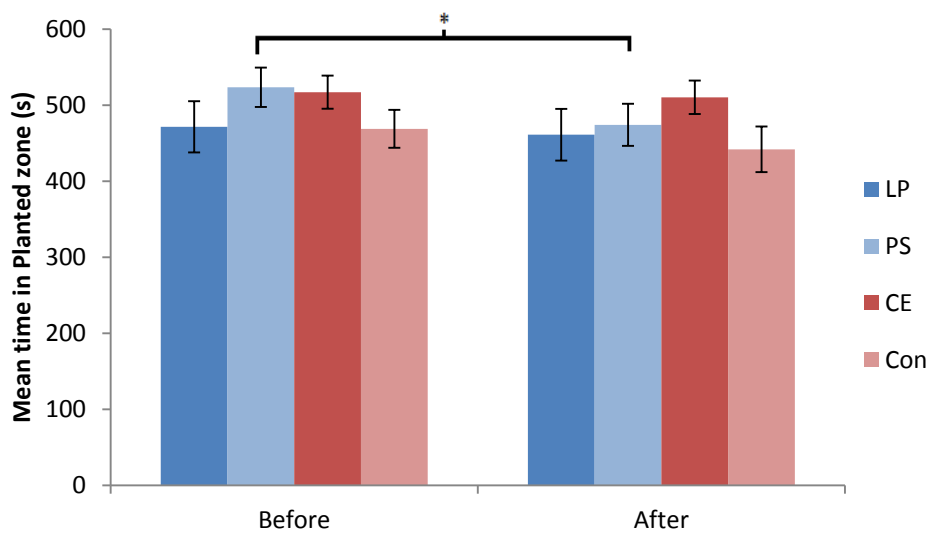


FIG. 2.4 Mean (\pm SE) time spent in the planted zone by conditioned fingerlings in the 10 min prior to the predator being revealed and the 10 min after the predator was revealed. Fingerlings were conditioned with one of four treatment; live predator (LP), predator scent (PS), conspecific extract (CE) and control (Con).

(*indicates $P < 0.05$).

All fish decreased their activity levels following exposure to a predator ($F_{1,78} = 19.29$, $P = <0.0001$) (Fig. 2.5), however, there was no effect of treatment ($F_{3,78} = 0.57$, $P = 0.639$) and no interaction between treatment and time ($F_{3,78} = 1.31$, $P = 0.277$). Fingerlings from the control treatment, the conspecific extract treatment and the live predator treatment all reduced activity levels after observing the predator (control; $F_{1,22} = 5.18$, $P = 0.033$, conspecific extract; $F_{1,18} = 8.05$, $P = 0.011$, live predator; $F_{1,20} = 12.71$, $P = 0.002$). Fingerlings from the predator scent treatment, however, showed no significant change in behaviour after the predator had been revealed ($F_{1,18} = 0.26$, $P = 0.615$).

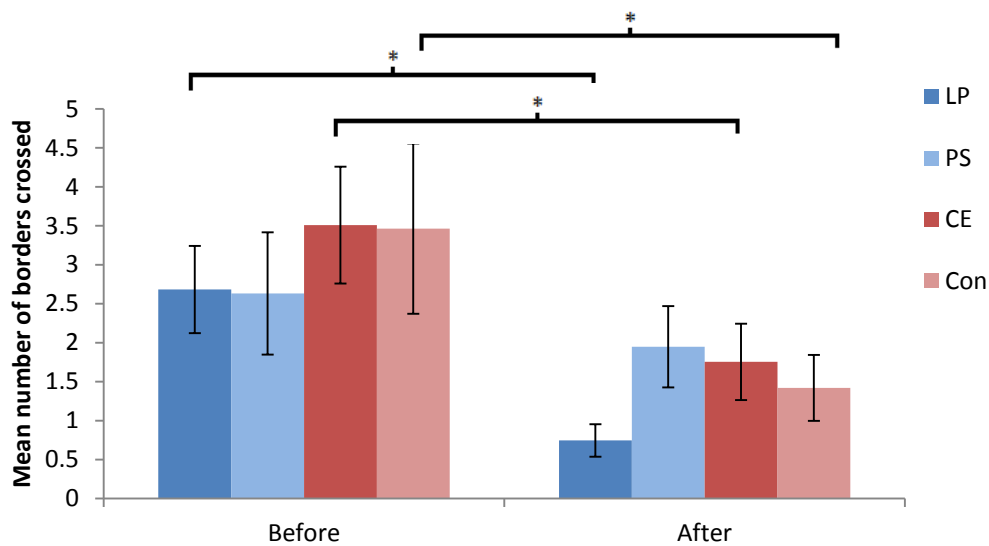


FIG. 2.5 Mean (\pm SE) activity levels of conditioned fingerlings in the 10 min prior to the predator being revealed and the 10 min after the predator was revealed (\pm SE). Fingerlings were conditioned with one of four treatments; live predator (LP), predator scent (PS), conspecific extract (CE) and control (Con). (*indicates $P < 0.05$).

2.5 Discussion

Conditioning *M. ambigua* fingerlings to recognise predators using predator cues resulted in a change in their anti-predator behaviour. The most dynamic change came from the fingerlings trained using olfactory cues only wherein predator scent was combined with conspecific extract. However, a graded response was seen from the other treatments, in what is probably a threat sensitive fashion (Lima and Dill, 1990). The fingerlings from different treatments showed a degree of behavioural plasticity which appears to be a function of prior experience (conditioning) and present predator threat. The immediate change in behaviour of the fingerlings after the presentation of a predator is indicative of their response to temporally specific information and a change in threat levels.

Whereas, changes in behaviour that were different from the control treatment and can be seen both before and after the reveal of the predator indicate a change in their background behaviour.

Chiefly, the fingerlings from each treatment spent the majority of their time in the planted zone, both before and after the predator was revealed. Whilst this response is not unexpected as structured habitats provide numerous advantages for fishes in lowland rivers (Crook and Robertson, 1999), it does reiterate the potential importance of stocking into areas near macrophytes to help improve survival immediately following release. However, the fingerlings from the predator scent treatment significantly increased use of the near zone after the predator was revealed and reduced use of cover. Furthermore, all treatments decreased activity after the predator was revealed, with the exception of the fingerlings from the predator scent treatment. It is likely the fingerlings from the predator scent treatment perceived a change in immediate threat levels and were performing predator inspections. Predator inspection is a well-known anti-predator tactic (Kelley and Magurran, 2007) and an effective way of gaining more information about the predator (Murphy and Pitcher, 1997; Brown and Godin, 1999), however it does place them closer to danger. Smith and Belk (2001) noted that risky behaviours, such as inspection, rely heavily on visual cues. Visual cues provide information that is temporally specific and specific for that predator. It is possible the fingerlings

from the predator scent treatment were investigating in an attempt to gather temporally specific information on the predator that is often acquired visually, such as hunger level, behaviour and size (Murphy and Pitcher, 1997).

The use of visual cues in predator recognition is likely to be highly context specific. In a situation where visibility is low, it is logical that chemical cues would be relied upon more heavily. Turbidity can interfere with the visual predator information that fish receive (Ferrari *et al.*, 2010b) and Hartman and Abrahams (2000) found chemical cues were used most when visual information was not available. Furthermore, they found that reliance on particular cues could be manipulated and the reduction of one source of information increased use of other cues. It certainly makes sense in such an important context as predator recognition and detection, that there is some degree of redundancy in the system which can act as a fail-safe if one sensory input is unreliable. Moreover, the use of two independent senses to detect predators likely acts as a reinforcer (McCormick and Manassa, 2008). The habitats preferred by *M. ambigua* are deep water (Crook *et al.*, 2001), slow, turbid lowland rivers with woody debris (Lintermans, 2007). In these situations use of chemical cues in predator recognition may be the best source of information. It is possible the fingerlings from the predator scent treatment, who received information from only one sensory input during the conditioning phase, had enough prior experience to cause them to investigate further, and make use of novel visual cues. It is likely that chemical cues function as early detection, yet visual cues are required for more temporally specific information. Thus, the different sensory modes may operate in a hierarchal fashion. Further-more, they may also have an additive effect when used in unison. A number of studies have found that the combination of visual and olfactory cues yield a stronger anti predator response than the individual cues in isolation (Smith and Belk, 2001; Mikheev *et al.*, 2006; McCormick and Manassa, 2008).

It is worth noting, that while the fingerlings conditioned with only CE and no predator cues did not show as much range in response as the fingerlings from the predator scent treatment, the trend in

area use was similar to that of the predator scent treatment. This suggests a degree of innate response to damaged conspecifics similar to that seen in ostariophysan alarm substances (ie. shreckstoff; Frisch, 1938). Innate responses to predator cues have also been found in rainbowfish *Melanotaenia duboulayi* (Castelnau 1878) embryos (Oulton *et al.*, 2013) and may be wide-spread in Australian native fishes.

The fingerlings from the control treatment showed a trend towards less use of cover both before and after the predator was revealed. Potentially, the conditioned treatments had changed their background behaviour as a result of their conditioning and exhibited a degree of anti-predator behaviour before the predator was revealed (such as increased vigilance). The prior history of the fingerlings is likely to impact on their anti-predator behaviours and background response. This pattern of response is often proportional to risk or graded, and dependant of factors such as frequency of risk (Foam *et al.*, 2005b; Mirza *et al.*, 2006), type of cue (Chivers *et al.*, 2001; Foam *et al.*, 2005a) and rearing environment (Berejikian *et al.*, 1999). For instance, fish from high background levels of risk have been found to show a reduced response to predator cues (Brown *et al.*, 2006). It is thought that this is in accordance with the risk allocation hypothesis, where prey has made a trade-off between anti-predator behaviour and lost opportunities in other behaviours such as foraging.

Greater levels of experience may help improve the ability to determine threat and lower the relative costs of anti-predator behaviour. For example, *P. reticulata* from high predation areas are better at ascertaining hungry predators than guppies from low predation areas (Licht, 1989). A combination of cues serves to provide an array of information on the predator and the risk it presents. Juvenile Atlantic salmon *Salmo salar* (L. 1758) use both visual and chemical information together to assess and respond to predators (Kim *et al.*, 2009) as do three-spined stickleback *Gasterosteus aculeatus* (L. 1758) and hatchery-reared pike *Esox Lucius* (L. 1758) (Lehtiniemi, 2005). The graded responses highlight the plasticity of behaviour, which is likely to be beneficial in natural environments, where

conditions change frequently. Moreover, the degree of individual variation seen in response to the training regime offers opportunity for managers to stock fish in a wide range of habitat types.

As *M. ambigua* are a popular target species with anglers and are produced in vast numbers for stocking into dams and impoundments, optimising post release survival seems both intuitive and economical. During the 2013 stocking season DPI Victoria stocked over 1 million *M. ambigua* (DEPI, 2013). Similar numbers have been stocked in Victoria in the years prior. The Native Fish Stocking Plan for 2013 in NSW stipulates a release target of well over 800,000 *M. ambigua* into dams and impoundments (DPI, 2012/2013). Despite this massive stocking effort, there is little data available regarding the success of stocked *M. ambigua*. Clearly this is a vital step to implement if one is to assess that efficacy of any stocking program. Recent statistics from the UK showed little or no improvement in salmon *S. salar* catch rates in stocked rivers (Young, 2013). In fact, there was some indication that stocking with naïve fish had a negative impact on salmon populations. These data highlights the fact that stocking needs to be very carefully planned and integrated with other management techniques such as habitat rehabilitation.

The improvements in behavioural repertoire of other hatchery reared species that have undergone pre-release training speaks to the success of this management strategy. For instance, Brown and Smith (1998) were able to condition hatchery reared juvenile rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) to recognise the chemical cues of a predator which caused an increase in anti-predator behaviour. Some studies have shown that pre-release training can increase post-release survival of hatchery reared fish. Experience with live food prior to release improved growth and survival of Atlantic salmon *S. salar* and sea trout *Salmo trutta* (L. 1758) (Czerniawski *et al.*, 2011) and predator recognition training improved predator recognition and survival of brook trout *Salvelinus fontinalis* (Mitchill 1814) (Mirza and Chivers, 2000). However, such studies are extremely rare in Australia and there are relatively few studies that have attempted pre-release training on an industrial scale. Similar research to this study performed by researchers for the Murray-Darling Basin

Authority (Hutchison *et al.*, 2012) showed promising results with pre-release training in other Australian native fish. An improvement in anti-predator behaviour was observed, and field trials for Murray cod *Maccullochella peelii* (Mitchell 1838) showed a significant improvement in post-release survival. However, our knowledge of the anti-predator behaviour of Australian native fish is still lacking and there are still many elements missing in the understanding of learned predator recognition in native species. The results presented herein suggest that both visual and chemical cues played a role in learned predator recognition in *M. ambigua*, although it appears olfactory cues elicit a greater range of response. The disparity in response patterns from the treatments suggests different information is acquired with different sensory modes. It is clear that much work remains to be done in this area.

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Chapter 3

Behavioural interactions between conditioned, hatchery-reared Golden Perch *Macquaria ambigua* and predatory spangled perch *Leiopotherapon unicolor* in a semi-natural enclosure

This chapter is formatted for publication in the Journal of Fish Biology. All figures and tables are embedded in text.

3.1 Abstract

The hatchery environment is known to suppress the development of anti-predator behaviours in fish. A lack of experience with predators prevents the enhancement of survival skills and is one of the key factors in the failure of many stocking efforts. In this study hatchery reared, juvenile golden perch (*Macquaria ambigua*), an Australian native freshwater fish, were conditioned with a range of predator cues combined with the odour of damaged conspecifics. Their anti-predator skills were then tested in a behavioural interaction trial with a predator at liberty in semi-natural conditions using a predatory spangled perch (*Leiopotherapon unicolor*), a common predatory fish of the Murray-Darling basin. The results showed anti-predator behaviour was enhanced by conditioning the fingerlings with predator cues. A range of responses was seen from fish trained with the different cues, with multiple cues eliciting the most intense response. Conditioning the fingerlings with both olfactory and visual cues resulted in an increased use of cover and conditioned fingerlings tended to approach the predator less often. These results suggest that pre-release training could be used as a strategy to improve post release survival in stocked golden perch.

Keywords: Golden perch, *Macquaria ambigua*, hatchery rearing, life skills training, olfactory cues, visual cues

3.2 Introduction

Assessing the level of threat posed by a predator and reacting to an appropriate degree allows prey to economise energy and time spent partaking in anti-predator activities. The threat sensitive hypothesis, coined by Helfman (1989), predicts that prey will match their response to the intensity of threat posed by a predator. To be able to do this well, however, prey must first be familiar with the cues associated with a predator.

The hatchery environment restricts development of anti-predator behaviours in fish (Olla *et al.*, 1998; Brown and Laland, 2001). This is largely due to the unnatural rearing environment, which does not allow for the full development of such behaviours owing to a lack of exposure. Predator recognition and anti-predator behaviours are known to have a significant learned component (Kelley and Magurran, 2003) which allows for a behavioural repertoire fine-tuned to suit contemporary conditions. Thus, prey fish that lack predator experience may be unable to recognise the cues associated with a predator and the level of danger they represent. This can lead to high levels of predation post-release in stocking situations. Pre-release training aims to overcome some of the behavioural deficits experienced by hatchery reared fish. One possible scenario is to condition them using predator cues paired with the cues of a damaged conspecific, thereby creating an aversive association and generating appropriate anti-predator responses.

Learned predator recognition often results from associations between damage released conspecific alarm cues and predator cues (Brown and Chivers, 2007). This mode of learning allows for a degree of behavioural plasticity in the face of shifting environmental conditions. Through these associations prey fish can learn to recognise a predator using different sensory modalities, both visual and olfactory. These different modalities vary in their attributes and the utility of these may depend on environmental conditions. In a situation where one sensory modality is compromised, compensation by other senses may occur. For example, under night light conditions, salmon *Salmo salar* (L. 1758) show a greater reliance on olfactory cues (Leduc *et al.*, 2010). Likewise fish may rely more heavily on

olfactory cues when vision is limited in other contexts, such as in low water clarity (Hartman and Abrahams, 2000). Visual cues, however, provide up to date information and details about the predator, such as size (Chivers *et al.*, 2001) and feeding status (Licht, 1989) that are not easily acquired through olfactory means. Thus each sense likely provides different information regarding the predator's identity and intentions.

The experience an individual has with predators and predator cues can play a significant role in anti-predator responses. Evidence shows fish from populations experiencing predation are more skilled at avoiding predation (Magurran, 1990; Magurran and Seghers, 1990). Differences in rearing environments results in differences in experience and behavioural repertoire. Hatchery rearing is well known to cause a suite of behavioural deficits in the fish produced when compared with wild counterparts (Olla *et al.*, 1998). For example, hatchery reared offspring of wild brown trout *Salmo trutta* (L. 1758) did not recognise the risk posed by predators (Álvarez and Nicieza, 2003). Predator recognition and anti-predator behaviour are known to have a significant learned component (for review see Brown and Chivers, 2007), thus experience is necessary for the development of a full complement of behaviours.

Rearing fish in hatcheries offers few opportunities to learn about or engage with predators, and consequently the hatchery reared fish are ill equipped to deal with the threat of predation appropriately after they have been released. However, efforts to improve the survival of hatchery reared fish have been investigated, and enhancing their behavioural repertoire has been identified as an area of potential development (Brown and Day, 2002). Brown and Smith (1998) conditioned hatchery reared juvenile trout *Oncorhynchus mykiss* (Walbaum 1792) to recognize a predators based on olfactory cues and successfully increased their anti-predator behaviour in the presence of the predator olfactory cues. Berejikian *et al* (2003) were able to improve the existing anti-predator response of Chinook salmon *Oncorhynchus tshawytscha* (Walbaum 1792) with just a single conditioning event. Similar conditioning tests have been successfully accomplished in a number of

studies, and recently, efforts have focused on measuring post release survival of conditioned fish. For instance, Lönnstedt *et al.* (2012) found conditioning reef damsel fish *Pomacentrus wardi* (Whitley 1927) with predator cues improved survival in their first 48hrs on a reef compared with those that were predator naïve. Interestingly, it appears experience with predators can occur even before fry have hatched, and paired predator cues and alarm cues can be recognised by embryos (Nelson *et al.*, 2013), which creates interesting possibilities for remedial behavioural steps to be applied early in the fishes life. Thus, the lack of predator interaction experienced within the hatchery environment can be improved by remedial methods such as pre exposure to predator cues and Olson *et al* (2012) recently showed predator conditioning can be achieved on a larger scale.

There is little evidence available concerning predator-prey interactions in large freshwater fish of Australia. This is surprising given the number of fish released in conservation and stocking programs throughout the country. Golden perch *Macquaria ambigua* (Richardson 1845) is a freshwater species of fish from the Murray-Darling region of Australia. They are often found in slow flowing, lowland areas (Lintermans, 2007) and are a prized target for recreational freshwater fishermen. During the 2013 stocking season Department of Primary Industries Victoria stocked over 1 million *M. ambigua* (DEPI, 2013). The Native fish stocking plan for 2013 in NSW planned a release target of well over 800,000 *M. ambigua* into dams and impoundments (DPI, 2012/2013). Despite extensive stocking efforts, little data is available regarding the success and survival of stocked *M. ambigua*. However, international studies suggest survival of released hatchery reared fish is generally very poor (see references within Olla *et al.*, 1998; Brown and Laland, 2001). Scientists funded by the Murray-Darling Basin Authority have recently performed predator conditioning experiments on a series of Australian freshwater fish and were able to enhance behavioural repertoire and more importantly, provide evidence of improvements in survival rate of conditioned fish post-release (Hutchison *et al.*, 2012). Similar research focusing on *M. ambigua* has the potential to comparably improve the stocking success and survival.

Here we examine the behaviour of conditioned juvenile, hatchery reared *M. ambigua* fingerlings in a behavioural interaction trial in semi-natural conditions with a predatory spangled perch *Leiopotherapon unicolor* (Günther 1859) at liberty. The fingerlings were conditioned with a range of predator cues paired with damaged conspecific cues prior to the trial. The aim of the study was to investigate the potential for predator conditioning to alter the behaviour and use of refuge of hatchery reared juvenile *M. ambigua* fingerlings in the presence of a predator.

3.3 Methods

3.3.1 Subjects and Housing

Subjects were *M. ambigua* fingerlings, approximately 8 weeks old when harvested and 3.5cm T_L . They were the first generation offspring of wild caught broodstock, produced by NSW Industry and Investments, Narrandera where they had been reared in outdoor dams. The fingerlings were transported overnight in oxygenated water, packed in boxes. Upon arrival at Macquarie University, Sydney, they were transferred into outdoor ponds (capacity 1530l) at a density of 50 fish per pond. The ponds were maintained under bird netting to avoid avian predation. They were fed frozen bloodworms 5 days a week and maintained in these outdoor ponds until the beginning of experiments.

The fish were then moved into tanks within a laboratory for conditioning prior to testing. The conditioning tanks measured 90cm long x 35cm wide and were filled to 35cm deep. Tanks were placed flush with the wall and all other sides of the tanks (excluding the top) were covered to minimise visual interaction and prevent undue stress on the fish from the activity of lab users. The room had a 12hrs light: 12hrs dark light cycle. Each aquarium was fitted with a filter (capacity 500l/hr), gravel and heater set at 16°C. The fish were placed in these tanks the day before conditioning started, to allow them to settle.

Three wild caught, mature *L. unicolor* with an approximate length of 15cm were used as predator fish. Spangled perch *L. unicolor* are an aggressive and active predator and are capable of catching and consuming several fingerlings in a day. Fish were kept individually in 60L tanks, with gravel substrate and a filter of 500l/hr capacity. The tanks were given a 1/3 volume water change each fortnight. The *L. unicolor* were fed on prawn 5 days a week prior to the experiment. They were fed only leading up to the conditioning phase, and not fed during the conditioning. Individuals were not used for more than 2 sequential trials to maintain motivation. After the conditioning, they were placed back in their housing tank and their feeding resumed.

3.3.2 Conditioning

A total of 16 replicate fish were conditioned per treatment. Fingerlings were conditioned in batches of 8 fingerlings per tank. Two batches of eight were conditioned per treatment to achieve the total of 16 fish per treatment. Four separate tanks were used to condition fingerlings. Each tank was designated to one of the 4 treatments. The fingerlings were moved into their conditioning tanks the day prior to the first exposure to allow them to settle sufficiently. Each tank was exposed 3 times per day to their treatment over three days (total of 9 exposures). Each exposure lasted 15min. A series of pilot studies suggested that this was the optimal training regime. This was done at between 9am – 10am, noon – 1pm and 3pm – 4pm. Chemical cues were administered slowly to avoid startling the fish unnecessarily. At the completion of the conditioning, each tank was drained and re-filled with de-chlorinated water.

3.3.3 Treatments

The ability of the fingerlings to learn to recognise predators was investigated using combinations of chemical and visual cues. The following four treatments were used:

3.3.3.1 Conspecific extract (CE)

To create conspecific extract (CE), whole carcasses of conspecifics (3000mg) were homogenized. This amount was extrapolated from the weight of conspecific skin used in studies in previous studies (Brown and Smith, 1998). As there is no information available on specific alarm pheromones in the study species, the decision was made to use the whole carcass, rather than just the skin. The 3000mgs of homogenised conspecific was then added to 100ml of distilled water and filtered through coarse filter paper (Advantec 6µm). Once this was done, more distilled water was added to bring the total up to 400ml. This was then poured into ice-cube trays, each cube containing 20ml of conspecific extract. The ice-cube trays were then frozen until use. The conspecific extract treatment received only 20ml of conspecific extract at conditioning times with no associated predator cues.

3.3.3.2 Predator scent plus conspecific extract

During this treatment (henceforth abbreviated to predator scent treatment) fingerlings received a combination of conspecific extract and predator scent only, with visual cues excluded. This is the standard approach to entrain anti-predator behaviour in fishes (Brown *et al.*, 2011). Water that contained the chemical signatures of the predator was used as predator scent. Immediately prior to exposure, 20ml of water was collected from a 60l tank containing a single *L. unicolor* who had been housed in the tank for an extensive period of time. This volume was similar to that used in studies by Brown & Smith (1998) but was reduced in volume to adjust for the extended housing of the *L. unicolor* in the tank prior to collection. The *L. unicolor* was fed a diet of frozen prawns 4 times per

week, thus there should be no dietary influence on the odour. Feeding was suspended during experimental periods. The tank had no water changes and was not cleaned for a week prior to the conditioning stage. At conditioning times, the scent was added directly to the conditioning tank and immediately followed by the addition of 20ml CE to the tank.

3.3.3.3 Live Predator (visual and chemical predator cues) plus conspecific extract

During this treatment (henceforth abbreviated to live predator treatment) a partition made from fine mesh (2mm²) was placed at 1/3 of the length of the tank creating two sections; a large section (62cm x 35cm depth x 35cm wide) and a small section (31cm x 35cm depth x 35cm wide). Water was able to pass freely through the partition allowing good circulation and facilitating movement of chemical cues between the compartments. An opaque partition placed next to the mesh was attached to a pulley allowing it to be lifted remotely. The fingerlings were placed in the large section of the tank. A *L. unicolor* was placed in the small section of the tank during each exposure. At each of the three daily exposures the opaque partition was lifted for a duration of 15min to allow visual contact with the predator. This was done in conjunction with the addition of 20ml CE. The predator was removed over night to prevent it attempting to escape.

3.3.3.4 Control

The control fish were not exposed to any cues. They were simply removed from the housing tank and placed in aquaria of the same dimensions as the other treatments for 15 min. In place of the nine exposures, the control group were simply left in the aquaria for the equivalent amount of time. The control group were representative of regular hatchery fish which have no conditioning prior to release.

3.3.4 Experimental Procedure

The testing was performed in a glass tank measuring 245cm (8ft) x 48cm deep x 61cm tall (Fig 3.1). This was divided into 2 by a barrier placed 65cm from one end to create a predator zone, where the predator could be released into the testing area with the fingerlings. The testing area was marked at every foot (30cm) to indicate the different 'zones'. Zones alternated open – planted from 1 to 6 up to the predator zone. Planted zones were furnished with plants and hides to provide extensive cover for the fingerlings to seek refuge in. Each planted zone contained 1 black, round pot (140mm diameter) with the base cut out and weights glued to the side to sink it, creating a cylinder large enough for the predator to swim through. Each planted zone also contained a small, black, square punnet (50mm at widest), similarly adjusted, that the predator was too large to comfortably fit into. In addition, each planted zone contained 5 large plastic aquarium plants. This combination of items gave dense cover and ample hiding places. The orientation of each of these items within their planted zone was changed between each trial, so the predator did not become too familiar with the layout. Open zones were left clear of plants and other items. A thin layer of pebbles covered the entire tank to reduce the reflectiveness of the glass base.

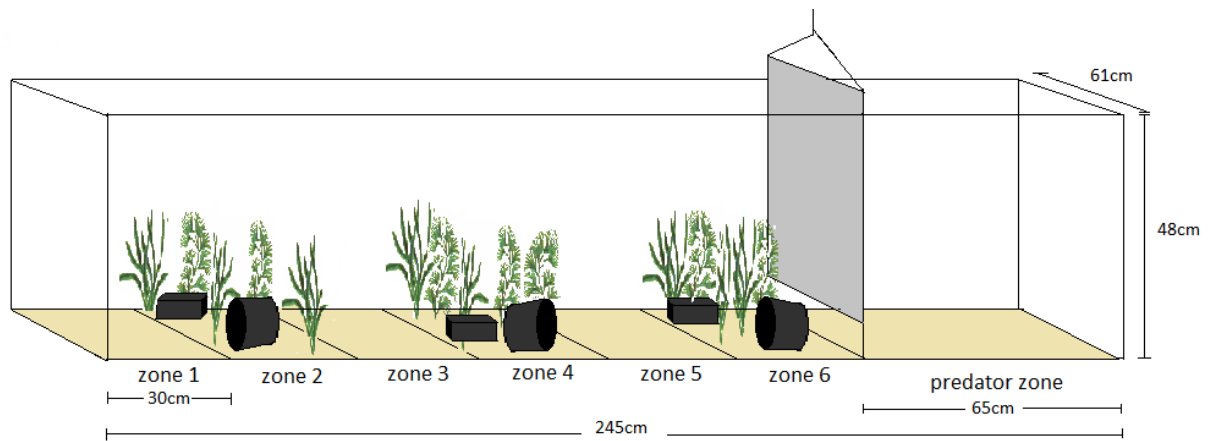


FIG. 3.1 Layout of the experimental tank. Zones 1, 3 and 5 were 'open' zones with no structures and zones 2, 4 and 6 were 'planted' zones, furnished with plants and refuges. The predator zone was separated by a Perspex barrier that could be lifted remotely.

Fingerlings were given 45 min to settle, before beginning a 30min test. The behaviour of both the fingerling and predator was observed. The use of refuge and open areas (termed area use) were recorded as well as activity, measured by counting borders crossed, and other distinct behaviours. Fingerling behaviours recorded included startle, approach, failed escape and escape. A startle was defined as a rapid, evasive movement, generally short in distance, away from the predator directed towards escape or avoidance. An approach was any distinct, investigatory movement towards the predator. A failed escape was when the predator caught and killed the fingerling, while an escape was when the predator captured but mishandled the fingerling resulting in its escape. Predator behaviours recorded were strike, approach, capture and failed capture. A strike was a distinct and fast lunge at the fingerling, approach was in this instance the predator approaching the fingerling. Capture refers to the predator capturing the prey and failed capture refers to the predator attempting but failing to capture prey. Escapes from the fingerlings and failed captures by the predator reflect the same event, as do failed escapes and captures. Ultimately, the total number of escapes (8) and failed escapes (5) remained too low to include in the analysis.

3.3.5 Data Analysis

Two observers recorded the data during the trial. One observer recorded the behaviour of the fingerling, the other recorded the predator's behaviour. Data was recorded using the EthoLog program (Ottoni, 2000). All data analysis was conducted using Statview 5.0.1 (SAS Institute Inc.).

Upon completion of the testing the data for planted zone use was squared and the data for open zone use and activity were log₁₀ transformed. Data for area use and activity was reduced to an average for each 10min of observation then analysed using Non parametric tests; Kruskal –Wallis and Mann-Whitney U tests for post-hoc pairwise analysis of treatments. Only approach data provided reliable results in the behaviours observed and thus, was the only distinct behaviour measured along with activity and zone use. Approaches by fingerlings across the entire 30min test were analysed as either having approached the predator one or more times (1) or not approaching (0) using a Binomial logistic model with post hoc pairwise comparisons performed using Wald tests. This was done as not all fingerlings made approaches towards the predator. Further to this, to assess the possibility that the predators behaved in a varied way for different treatments, approaches made by the predator were examined using a one way ANOVA. No effect of treatment was found ($F_{3,58} = 0.82$, $P = 0.486$) and the post hoc tests (Fisher's PLSD) showed no significant differences in predator behaviour between pairs of treatments. Thus we can be certain that predator behaviour was consistent across treatments.

3.4 Results

Kruskal-Wallis tests were conducted to evaluate differences in use of cover by conditioned fingerlings during a behavioural interaction trial with a predator at liberty in semi-natural conditions. An overall interaction between treatment and area use was found for both planted zone ($H(3) =$

12.27, tied $P=0.002$) and open zone use ($H(3) = 10.79$, tied $P=0.004$) (Fig 2). Pairwise comparisons were then performed using Mann-Whitney U-tests to distinguish differences in treatment medians of planted zone use (Table I). The fingerlings from the CE and live predator treatments made more use of cover, whilst the fingerlings from the control and predator scent treatments recovered over time, reducing their use of cover (Fig 3.2).

Table I. Pairwise comparisons of treatments where a significant difference was found between treatment medians in use of planted zone.

Treatment x treatment	U	Z	Tied- P
CE x control	(3) 634.50	-2.07	0.023
CE x predator scent	(3) 578.50	-2.05	0.025
control x live predator	(3) 517.00	-2.79	0.002
live predator x predator scent	(3) 1014.00	-2.75	0.002

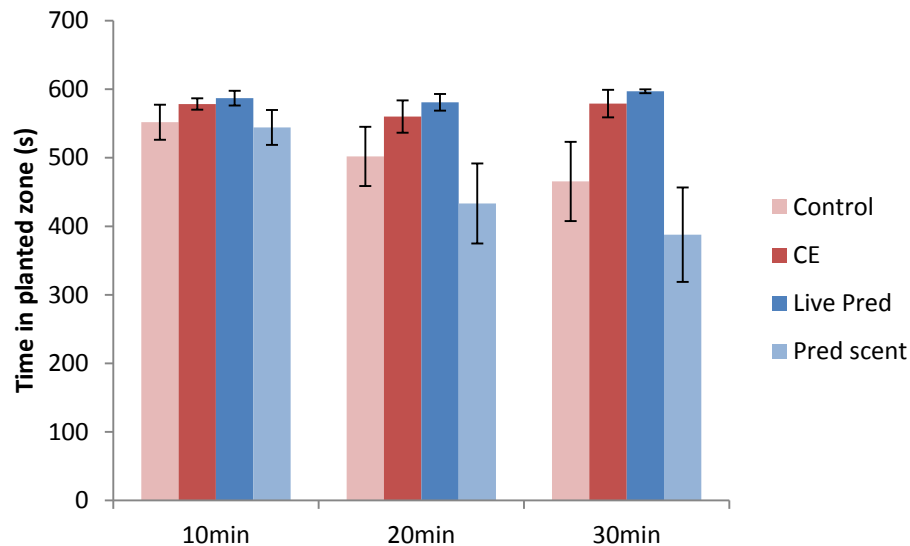


FIG 3.2. Area Use. Mean (\pm SE) time spent in the planted zone by conditioned fingerlings. Fingerlings were conditioned with one of four treatments; live predator (LP), predator scent (PS), conspecific extract (CE) and control (Con).

A Kruskal-Wallis test was also performed to examine the relationship between activity levels and conditioning treatment. Treatment was found to have a significant effect on the activity levels of the fingerlings ($H(3) = 8.55$, tied $P = 0.014$) (Fig. 3.3). Pairwise post hoc comparisons using Mann-Whitney U-tests indicated the fish in the live predator treatment were less active than the fish in both the control treatment ($U(3) = 547.50$, $z = -2.49$, Tied $P = 0.005$) and the predator scent treatment ($U(3) = 517.00$, $z = -2.28$, Tied $P = 0.010$).

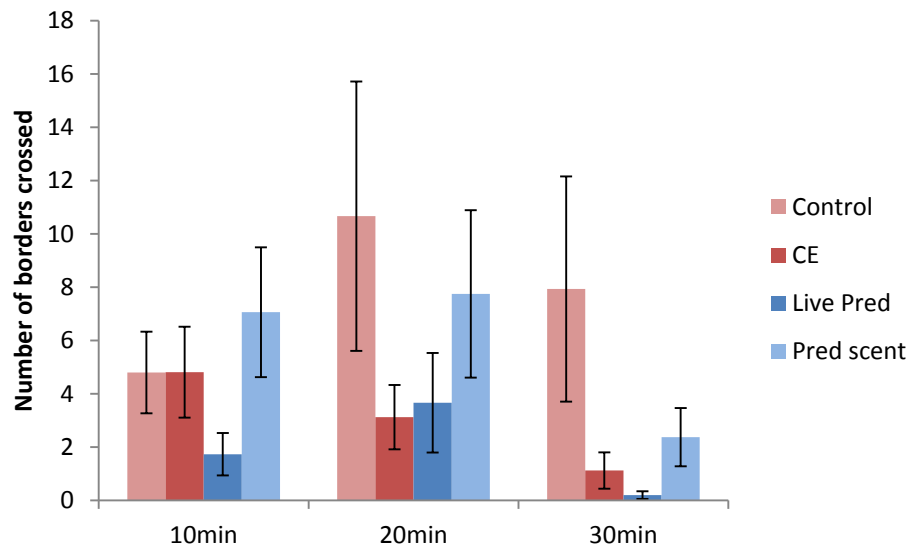


FIG 3.3 Mean (\pm SE) activity levels of conditioned fingerlings. Fingerlings were conditioned with one of four treatment; live predator (LP), predator scent (PS), conspecific extract (CE) and control (Con).

Approaches of the fingerlings towards the predator were also examined (Fig. 3.4), with fingerlings recorded as either having approached the predator one or more times (1) or not having approached the predator for the entire time period (0). The results of a Binomial logistic model show there was an overall effect of treatment on the tendency to approach a predator ($df_{1,3}$, Wald $\chi^2 = 9.466$, $P = 0.024$). Pairwise post hoc Wald tests indicate a significantly greater tendency to approach the predator in fingerlings from the conspecific extract treatment than fingerlings from the predator scent treatment ($P = 0.049$) and live predator treatment ($P = 0.010$), and from fingerlings from the control treatment than fingerlings from the predator scent treatment ($P = 0.016$) and the live predator treatment ($P = 0.002$) (Table II).

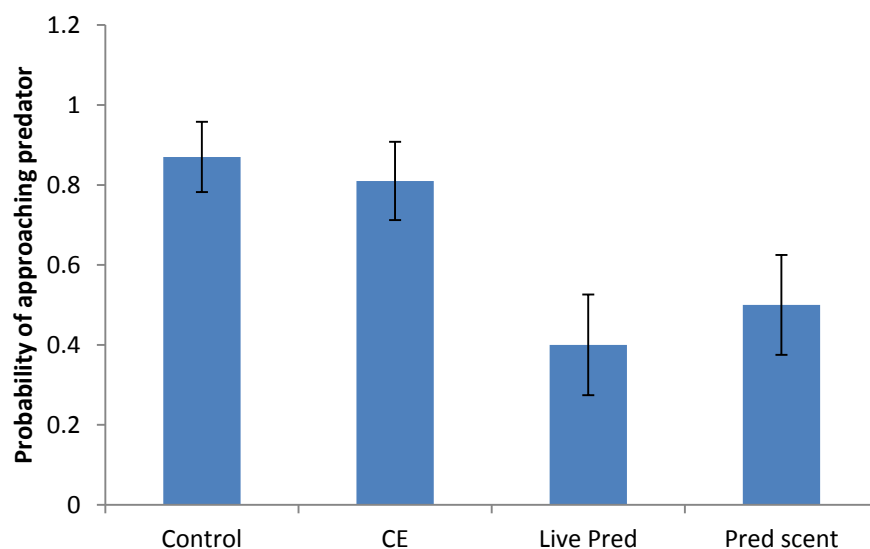


FIG 3.4. Probability (\pm SE) of fingerlings in each treatment approaching the predator. Fingerlings were conditioned with one of four treatments; live predator (LP), predator scent (PS), conspecific extract (CE) and control (Con).

Table II. *P*-values of pairwise post hoc analysis of Walds test for approaches made by the fingerlings towards the predator.

<i>P</i> – Values	Control	PS	LP
CE	0.680	0.049	0.010
Control	-	0.016	0.002
PS	-	-	0.574

3.5 Discussion

Predator recognition was enhanced by conditioning *M. ambigua* fingerlings with predator cues. A range of responses was seen from the different cues, with multiple cues eliciting the greatest range of responses. Conditioning the fingerlings with both olfactory and visual cues (live predator treatment) resulted in a response to the predator that consisted of a greater use of cover and fewer approaches towards a predator. Use of refuge is a commonly recognised response to avoid predation (Everett and Ruiz, 1993). It is possible the fingerlings trained with multiple cues were the most experienced with predators and thus reacted the most intensely. This may be due to an increase in information available allowing the fingerlings to make a more informed decision. For example, Manassa *et al.* (2013) showed the addition of visual cues to olfactory cues caused a dramatic change in anti-predator behaviour in anemonefish *Amphiprion percula* (Lacepède 1802) when compared to olfactory cues alone. Multi modal cues may help promote accuracy in predator encounters and behavioural decisions (Smith and Belk, 2001; Ward and Mehner, 2010) as more detail is available for decision making. The combination of visual and olfactory cues can also reinforce and have an additive effect, resulting in a stronger response (McCormick and Manassa, 2008).

Different information is gathered from each of the sensory modes, each modality having a distinctive utility. Olfactory cues may provide an early warning of a predator in the vicinity (Chivers *et al.*, 2001) and they may provide details of the predators recent diet (Smith and Belk, 2001). Visual cues, however, provide more reliable and accurate information that is temporally specific (Licht, 1989; Murphy and Pitcher, 1997; Smith and Belk, 2001). Thus, reliance on particular sensory modalities is likely to be context specific. The habitats favoured by *M. ambigua* are generally associated with woody debris (Crook *et al.*, 2001) and may also be highly turbid (Lintermans, 2007). In situations where visibility is compromised, olfactory cues may be relied upon more heavily (Hartman and Abrahams, 2000; Leduc *et al.*, 2010). In this experiment, however, visual cues were not limited by

water clarity and this may account for a greater reliance on visual cues. The addition of visual cues to olfactory cues provides more detailed and up to date information on threat levels. For instance, guppies *Poecilia reticulata* (Peters 1859) can differentiate between a satiated or hungry predator (Licht, 1989). These visually acquired details are important in accurately assessing the threat posed by a predator. For instance, when slimy sculpins *Cottus cognatus* (Richardson 1836) could visually assess a predator they were able to adjust their anti-predator response to the level of risk posed by a predator, in this instance, by size (Chivers *et al.*, 2001). The reactions seen from the different treatments were graded, demonstrating a plasticity in behavioural response that was likely threat sensitive and relative to the degree of perceived threat.

Fingerlings conditioned using only CE showed a significant increase in use of cover compared with the fingerlings from the control treatment, although not as distinct as that seen from the fingerlings from the live predator treatment. This suggests that there is a degree of innate response to the odour of damaged conspecifics. Examples of dramatic anti-predator reactions to the odour of damaged conspecifics are numerous (Chivers and Smith, 1998; Brown and Chivers, 2007) and form the foundation for many pre-release training methods. It is possible that the fingerlings conditioned with only CE were reacting in a generalised fright reaction, as they had no associated predator cue during conditioning. Holmes and McCormick (2011) showed using varied concentrations of alarm cues elicited a threat sensitive response in newly settled coral reef fish *Pomacentrus amboinensis* (Bleeker 1868). Indeed, even low concentrations of alarm cue, that are insufficient to elicit a dramatic response, can cause fish to increase vigilance to secondary cues, such as visual cues (Brown *et al.*, 2004). Regardless of an accompanying predator cue, an innate anti-predator reaction to the odour of damaged conspecifics is likely to provide a survival benefit to prey fish.

Interestingly, the fingerlings from the CE treatment, along with those of the control, also showed a greater tendency to approach the predator than fingerlings from the conditioned treatments. Whilst this increase in proximity to the predator may seem a counter-productive and risky behaviour, this

could be viewed as predator inspection, where the fingerlings attempt to gain more information regarding the nature of the threat. Fingerlings from the conditioned treatments on the other hand, were more experienced with the range of cues provided and thus were more equipped to estimate the potential threat posed by the predator. These behaviours were likely a response to recent perceived threat levels that the fingerlings adjusted their behaviour to match, as is predicted by the threat sensitive hypothesis (Helfman, 1989).

The difference in experience with predators between treatments resulted in a range of responses, highlighting the role that experience plays in anti-predator behaviours. Typically, the hatchery environment is geared toward productivity, and little concern is given to the behavioural development of the fingerlings reared within. In situations where fingerlings are being raised for eventual release in stocking programs it would be prudent to make efforts to improve survival post-release. The effectiveness of life skills training has been proven in a number of laboratory trials. For instance, hatchery reared juvenile Japanese flounder *Paralichthys olivaceus* (Temminck & Schlegel 1846) are better able to avoid capture by crabs if they have had prior experience with crabs (Hossain *et al.*, 2002). Likewise, experience with use of refuge has been demonstrated to improve post release survival of hatchery reared white seabream *Diplodus sargus* (L. 1758) (D'Anna *et al.*, 2012). By facilitating predator experience in a non-lethal way, there exists the possibility for pre-release training to improve the behavioural response of hatchery reared *M. ambigua* after release. More recently, endeavours have been made to show that conditioning of hatchery reared fingerlings can be achieved on a larger, industrial scale (Olson *et al.*, 2012). However, to date, the use of life-skills training in Australia has been limited. Researchers funded by the Murray-Darling Basin Authority performed conditioning experiments on freshwater Australian native species of fish and found they were able to improve anti-predator response in Murray cod *Machullochella peelii* (Mitchell 1838), silver perch *Bidyanus bidyanus* (Mitchell 1838) and catfish *Tandanus tandanus* (Mitchell 1838) (Hutchison *et al.*, 2012). Furthermore, the *M. peelii* experienced greatly improved survival rates once released.

The potential for use of pre-release training in Australian species exists but is likely to be highly species specific, thus further research efforts need to be tailored to the species examined. The results presented herein highlight the plasticity of behaviour and the importance of experience in developing anti-predator behaviour. The results of this study suggest that conditioning *M. ambigua* fingerlings to recognise novel predators is possible and could serve to improve anti-predator behaviour and subsequently improve post-release survival of hatchery reared fingerlings.

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Chapter 4

Predator conditioning in hatchery reared Australian bass *Macquaria novemaculeata*: The roles of visual and olfactory predator cues in learned predator recognition

This chapter is formatted for publication in the Journal of Fish Biology. All figures and tables are embedded in text.

4.1 Abstract

Fish reared in hatcheries are removed from the pressures of predation and do not get the opportunity to learn valuable predator recognition skills. The use of pre-release training aims to overcome these behavioural deficits and facilitate learned predator recognition through associative learning with the scent of damaged conspecifics. Here we investigated the relative roles of different modalities of predator cues on learned predator recognition in a laboratory environment on juvenile hatchery reared Australian bass *Macquaria novemaculeata*. Conditioning the fingerlings using predator cues elicited a change in behaviour that was mediated by conditioning treatment and behavioural assay used, suggesting a threat sensitive approach to learning and predator recognition. Conditioning the fingerlings exclusively with olfactory predator cues tended to elicit a more intense response than conditioning with only visual cues from the predator or both visual and olfactory cues. However, this was dependant on the test performed and a graded response was observed in the other treatments that varied in intensity. From these results it can be concluded that conditioning *Macquaria novemaculeata* fingerlings using predator cues is sufficient to cause a change in anti-predator behaviour and could potentially be used in behavioural remediation programs. However, the plasticity of the response to training suggests such attempts would need to be highly specific for this species and to the environment in which they are to be liberated in.

Key words: Australian Bass, *Macquaria novemaculeata*, hatchery rearing, predator cues, learned predator recognition, threat sensitive.

4.2 Introduction

The first step in anti-predator behaviour is to recognise and assess the threat of a potential predator (Kelley and Brown 2011). Doing so correctly is essential for the continued survival of an individual. In fish, this is done using a range of cues from the predator, including olfactory and visual cues. It is understood that recognition of predators by fish is largely a learnt skill, that develops through experience with predators and with the cues they transmit (Brown et al. 2011). Through learned predator recognition, each individual's experience can shape a behavioural repertoire that is unique to their environment. The intensity of anti-predator responses can then be moderated to match the level of threat perceived, as is predicted by the threat sensitive hypothesis (Helfman 1989).

Olfactory cues are a commonly employed source of information for fish and one of their utilities lies in the rapid dispersal of these cues through the aqueous environment (Wisenden 2000). While olfactory cues may serve as an early warning, visual cues are better at providing temporally specific information on the threat posed by a predator (Chivers et al. 2001). For instance, guppies *Poecilia reticulata* (Peters 1859) are able to distinguish between a hungry predator and a satiated one (Licht 1989), visually acquiring information specific to the level of threat posed by the predator at that time. Similarly, Brown and Warburton (1997) found both the shape and movement were key cues in threat detection. Information garnered from multiple sensory modalities contributes to behavioural decisions. The relative importance of the different modalities varies and can be manipulated experimentally. For example, Hartman and Abrahams (2000) sensory compensation model showed reliance on other sensory modalities can be increased when the primary one is ineffective. If one cue is unavailable, such as in situations of low visibility, or compromised, the other cues may be relied upon more heavily (eg lateral line or chemical information).

The relative roles of different predator cues have often been examined in a threat sensitive context. For instance, Chivers *et al* (2001) found slimy sculpins *Cottus cognatus* (Richardson 1836) responded to predators in a threat sensitive way only when presented with visual cues, and in a non-threat

sensitive way when presented with olfactory cues. McCormick and Mananssa (2008) however, found the response of coral reef fish *Asterropteryx semipunctatus* (Rüppell 1830) to either olfactory or visual cues alone was the similar in magnitude, but combining the cues created an additive effect and elicited a stronger response.

In addition to adjusting to threat levels, anti-predator behaviour can be shaped by previous experience. A number of studies have shown anti-predator behaviour and predator recognition is enhanced in predator experienced individuals (for reviews see Kelley and Magurran 2007; Ferrari et al. 2010). Brown and Smith (1998) were able to condition hatchery reared juvenile trout *Oncorhynchus mykiss* (Walbaum 1792) to react in an anti-predator response to the scent of predatory pike through associative learning using conspecific skin extract. Likewise, conditioning juvenile coral reef damselfish *Pomacentrus wardi* (Whitley 1927) fish to recognise the cues of predators can enhance their survival over those that had no experience with predator cues (Lönnerstedt et al. 2012).

The pairing of alarm cues and predator cues has been utilised as a tool for facilitating predator recognition and enhancing anti-predator behaviour during pre-release training of hatchery reared fish (Brown and Laland 2001) and aims to overcome some of the behavioural deficits experienced by hatchery reared fish as a consequence of their rearing environment (Olla et al. 1998). Predator recognition training has been used with the intent of improving post release survival of hatchery fish, and field-enclosure studies have shown it can improve survival (Mirza and Chivers 2000), but few have attempted training on an industrial scale. Recent work from researchers for the Murray-Darling Basin Authority has shown promising results in pre-release behavioural modification in silver perch *Bidyanus bidyanus* (Mitchell 1838), Murray cod *Maccullochella peelii* (Mitchell 1838) and freshwater catfish *Tandanus tandanus* (Mitchell 1838) (Hutchison et al. 2012). Indeed, the survival of *M. peelii* after stocking into rivers was significantly improved by the anti-predator training they received prior to liberation. However, predator recognition and life skills training have been relatively unstudied in Australian freshwater fishes.

Australian bass *Macquaria novemaculeata* (Steindachner 1866) are a freshwater species of fish found in coastal drainages along the south-east coast of Australia, preferring rocky pools with aquatic vegetation (Allen et al. 2002). *M. novemaculeata* are a popular target for recreational fishers and are stocked in large numbers throughout NSW, Qld and Vic. They are successfully bred in hatcheries and are produced in large numbers along the east coast. The planned releases for the 2012/2013 period by the NSW Department of Primary Industries (DPI) estimated over 280,000 would be released into dams and lakes (DPI 2012/2013). They are a catadromous species and require access to the sea in order to reproduce. Subsequently, landlocked water bodies allow for very controlled stocking situations where released *M. novemaculeata* cannot reproduce and create a self-sustaining population. However, for the same reasons, dams and weirs can obstruct breeding, and natural, inland populations may be restricted (Harris 1988). Although they are commonly stocked, little is known about the success of juvenile *M. novemaculeata* post release. Furthermore, there is an absence of information regarding how they learn about and recognise potential predators. Indeed behavioural studies focusing on *M. novemaculeata* are limited and little information regarding their schooling behaviour and anti-predator behaviours is available. Here we investigate the use of predator cues by *M. novemaculeata* in predator recognition. Three main questions were addressed; 1) can predator conditioning alter the anti-predator behaviour of small groups of *M. novemaculeata*, 2) what modalities, or combination of, during conditioning elicit the most intense reaction in response to different predator cues.

4.3 Methods

4.3.1 Subjects and Housing

Young of the year *M. novemaculeata* fingerlings of around 5cm total length (L_T), produced under the Hatchery Quality Assurance Scheme (HQAS) and reared in outdoor dams were used. These fingerlings were the first generation offspring of wild caught broodstock. Fish were packed in

oxygenated water and transported overnight in polystyrene boxes. Upon arrival at Macquarie University, Sydney, the fingerlings were transferred into outdoor ponds (capacity 1530 l) at a density of 55 fish per pond. The ponds were maintained under bird netting to avoid avian predation. They were maintained under shade cloth at ambient temperature, averaging between 13.5°C and 22°C. During the first week, the fingerlings were weaned onto flake food by using bloodworms mixed with flake. They were fed flake food 5 days a week and maintained in these outdoor housing ponds until the beginning of experiments.

Fingerlings were conditioned in batches of 16 (4 schools of 4 fingerlings) and pseudorandomly assigned to one of four treatments outlined in detail below. Because the behaviour of fish within a shoal is dependent on the behaviour of their fellow school mates, each school of four fingerlings represented one replicate. Each treatment had a total of 12 replicate schools.

Three wild caught, mature, spangled perch *Leiopotherapon unicolor* (Günther 1859) were collected from the wild for use as predators. The predators were shipped to the university in plastic bags, augmented with pure oxygen, inside polystyrene crates. *L. unicolor* were housed individually in 60 l aquaria, lit overhead by fluorescent lights with a 12 hr light: 12hr dark light cycle. Each aquarium was fitted with a filter (capacity 500l/hr), gravel and heater set at 18°C. They were fed a diet of frozen prawns four times per week. Feeding was suspended during experimental periods to enhance hunting motivation. The predator's housing tank had no water changes and was not cleaned for a week prior to the conditioning stage. A litre of water was extracted and refrigerated for the predator scent, then aliquots were brought up to room temperature before use.

4.3.2 Conditioning

The fingerlings were moved into tanks within a laboratory for conditioning prior to testing. The conditioning tanks measured 90cm x 35cm and were filled with water to 35cm depth. Tanks were placed flush with the wall and all other sides of the tanks (excluding the top) were covered to

minimise visual interaction and prevent undue stress on the fish from the activity of lab users. The room had a 12hrs light: 12hrs dark light cycle. Each aquarium was fitted with a filter (capacity 500l/hr), gravel and heater set at 18°C. The fish were placed in these tanks the day before conditioning started to allow them to settle. They were then exposed to predator cues three times per day, over 2 days, for a total of 6 exposures according to their treatment. This level of training was determined by an extensive series of pilot studies and appeared to induce the greatest response from the fish. We utilised a common classical conditioning procedure whereby a variety of cues were associated with conspecific extract (CE). CE is known to illicit innate anti-predator responses in a wide range of fish and induces a conditioned response when paired with other cues (Brown and Chivers 2007).

4.3.3 Conspecific Extract (CE)

CE was generated by homogenising whole conspecifics and passing the liquid through a filter (see Chapter 2 Methods 2.3.3.1 for details).

4.3.4 Treatments

4.3.4.1 Visual cues combined with CE

During this treatment (henceforth abbreviated to visual cues treatment) the fingerlings were exposed to visual cues from the predator in conjunction with CE, in the absence of predator scent. This was done by keeping the fingerlings in a tank covered with black plastic on three sides to limit any other visual cues. On one short side, a tank of the same dimensions containing a predator was placed flush with the fingerling tank. An opaque Perspex barrier was placed between the two tanks and was attached to a remote pulley that allowed it to be lifted, providing visual contact between the two tanks. At the conditioning times, the predator was gently guided to the end of its tank

closest to the fingerlings and contained there by placement of a clear barrier at 30cm away from the end of the tank. The visual barrier between the two tanks was lifted and the CE added to the fingerlings tank at the end nearest the predator. The barrier remained up for 15min. At the end of the 15min, it was lowered and the predator was released by removing the clear partition until the next conditioning interval. Prior to conditioning of the fingerlings, trials were conducted with dye in all tanks to ensure water spread evenly throughout the tank within a 3min time frame. The tanks were thoroughly cleaned and refilled before use with fingerlings. At the completion of each conditioning the fingerlings tank was drained completely, cleaned and refilled with de-chlorinated tap water.

4.3.4.2 Predator scent combined with CE

During this treatment (henceforth abbreviated to predator scent treatment) fingerlings received a combination of conspecific extract and predator scent only, with visual cues excluded. Water that contained the chemical signatures of the predator was used as predator scent. Immediately prior to exposure, 20ml of water was collected from the *L. unicolor* tank. This volume was similar to that used in previous studies (Brown & Smith (1998) but was reduced in volume to adjust for the extended housing of the *L. unicolor* in the tank prior to collection. The scent was added directly to the conditioning tank and immediately followed by the addition of 20ml CE to the tank. At the completion of each conditioning the tank was drained completely, cleaned and refilled with de-chlorinated tap water.

4.3.4.3 Live predator (visual and chemical predator cues) combined with CE

During this treatment (henceforth abbreviated to live predator treatment) a live predator was placed behind a barrier within the fingerlings tank. A partition made from fine mesh (2mm²) was placed at 1/3 of the length of the tank creating two sections; a large section (62cm x 35cm depth x 35cm wide) and a small section (31cm x 35cm depth x 35cm wide). The predator was placed in the

small section of the tank whilst the fingerlings were placed in the large section. Water was able to pass freely through the partition allowing good circulation and facilitating movement of chemical cues between the compartments. An opaque partition placed next to the mesh was attached to a pulley and lifted at each exposure for a duration of 15min to allow visual contact with the predator. This was done in conjunction with the addition of 20ml CE. The predator was removed over night to prevent it attempting to escape. At the completion of each conditioning the tank was drained completely, cleaned and refilled with de-chlorinated tap water.

4.3.4.4 Control

The control fish were not exposed to any cues. They were simply removed from the housing ponds and placed in aquaria of the same dimensions as the other treatments. In place of the six exposures, the control group were simply left in the aquaria for the equivalent amount of time. The control group were representative of regular hatchery fish which have no conditioning prior to release. At the completion of each conditioning the tank was drained completely, cleaned and refilled with de-chlorinated tap water.

4.3.5 Experimental Procedure

Testing began the day following conditioning. Each day the schools were exposed to four different tests (details outlined below) and their behavioural responses monitored. Each school was exposed to the four tests sequentially with each school beginning at a different test to control for exposure order. In all instances the fish were placed in the testing tank or pool and allowed 20 min to settle before recording began. They were then recorded for 20 min before the introduction of any predator cues. The predator cue was then added and the recording continued for a further 20min. Total test time for each test was one hour.

4.3.6 Area use tests

The area use tests involved testing preferences for planted or uncovered, open areas before and after addition of a predator stimulus, using either predator scent or the live predator.

4.3.6.1 Area use following exposure to a live predator

The tank used for measuring area use in response to a live predator measured 92cm x 51cm, filled to a depth of 31cm (Fig 4.1) and consisted of two compartments, separated by a Perspex partition placed at approximately 1/3 of the length of the tank (30cm). The smaller section was referred to as the predator zone. The partition was clear and had small holes drilled in it to allow some water movement, thus allowing for both chemical and visual contact of fish on either side. Gravel was placed on the bottom of the predator zone, blocking the bottom and edges of the partition to prevent fingerlings from entering the predator zone. A small pump (capacity of 150L per hour) was also placed in the predator zone to ensure circulation of the predator scent. The remainder of the tank was divided in to three 'zones', marked on the underside of the tank with black marker. The area closest to the predator was termed the 'near' zone (30cm x 50cm). Behind this was designated the 'planted' zone and contained four plastic plants evenly spaced within the zone (30cm x 25cm). The other zone was designated the 'open' zone which remained devoid of structure (30cm x 25cm). This layout enabled us to distinguish any preferences for the planted zone as being distinct from the distance from the predator. The position of the planted zone and the open zone alternated with each test to control for side preferences. Entering the near zone was likely to reflect predator inspection whilst use of the planted zone was likely to indicate refuge use. In addition to the clear Perspex partition, an opaque barrier was also placed in the tank visually separating the predator from the fingerlings. This was attached to a pulley so it could be lifted remotely allowing visual

contact between the fingerlings and predator. The solid Perspex barrier also prevented the scent leaving the predator zone prior to exposing the predator to the prey.

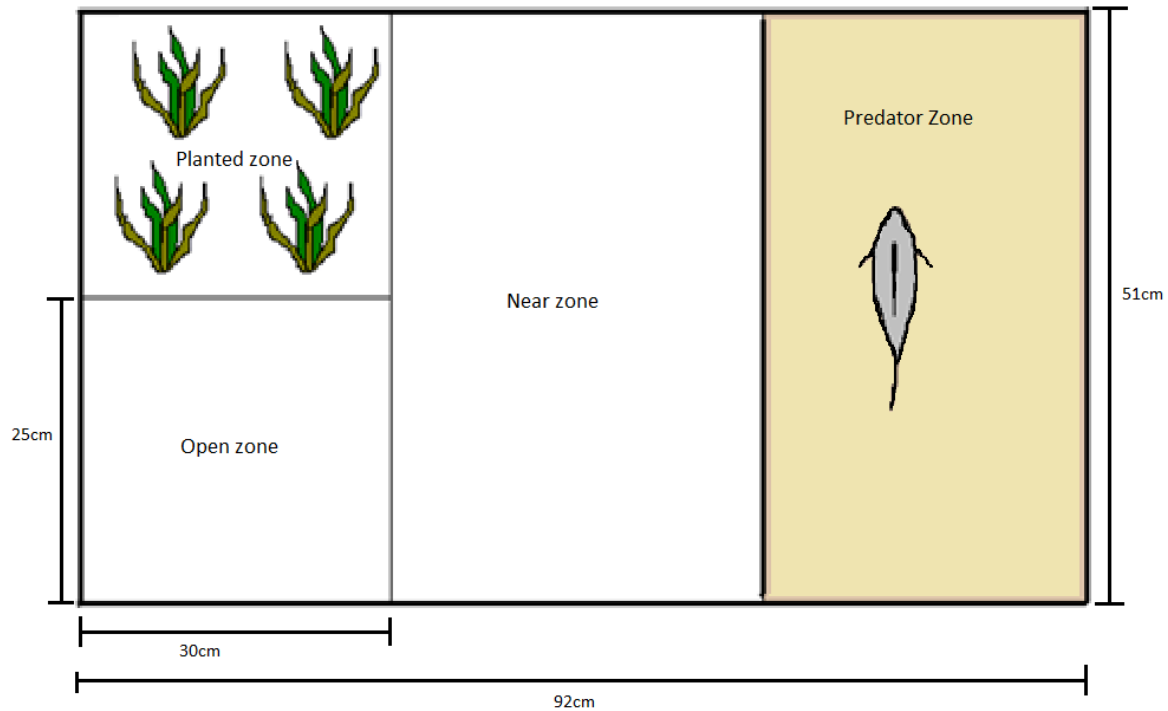


FIG. 4.1 A diagram of the experimental tank used for the area use; live predator test. Measurements 92cm long x 51cm wide, filled to a depth of 31cm. Gravel was placed on the base of the predator zone and the planted zone was furnished with four plastic aquarium plants.

Each school was tested by placing them in the near zone, allowing them to settle and recording their behaviour on an overhead camera before and after the predator was revealed. Their use of the different zones was recorded by taking frame shots every 15 s from the recording and counting the number of fingerlings in each zone. The relative use of different zones was termed area use. The change in number of fish between zones from one frame shot to the next was recorded as a proxy measure for activity levels of the fingerlings.

4.3.6.2 Area use following exposure to predator scent

A tank measuring 86cm x 46cm, filled to a depth of 32cm was used for this experiment. It was lit overhead by fluorescent lights and maintained at room temperature (approximately 19°C). The base of the tank was glass atop a white base marked into quarters. The tank was covered in thick white plastic to reduce disturbances from outside of the tank and to make the fingerlings more visible to the observing researchers.

The tank was split into 4 equal sized zones (Fig. 4.2). Two of the diagonally opposed zones were designated planted and the other two were open. Each planted zone had four plastic aquarium plants in it to provide dense cover for the fish to hide in. The open zones had no cover. A tube was attached in the centre of each of the short sides of the tank, through which predator scent could be delivered. In each planted zone, next to the tubing, a small pump (capacity of 150L per hour) was placed to ensure circulation of the predator scent (Fig. 4.2). The school of four fingerlings were introduced to the test tank and allowed to settle. Their behaviour was then recorded via a video camera mounted above the tank. After the initial recording period 10ml of predator scent (see 'treatments' above for definition) was added to each of the two tubes and flushed with air to ensure all the scent had been delivered. Recording continued for a further 20min after the addition of predator scent. At the end of the experiment the fingerlings were removed and the tank emptied completely, cleaned and refilled prior to the next group.

Data regarding the location of the fish was recorded from video frame shots taken every 15 s. From these frame shots, the number of fish in either the planted or the open zone was recorded. The change in number of fish from the planted to open zones between each frame shot was also recorded as a proxy measure for activity levels of the fingerlings.

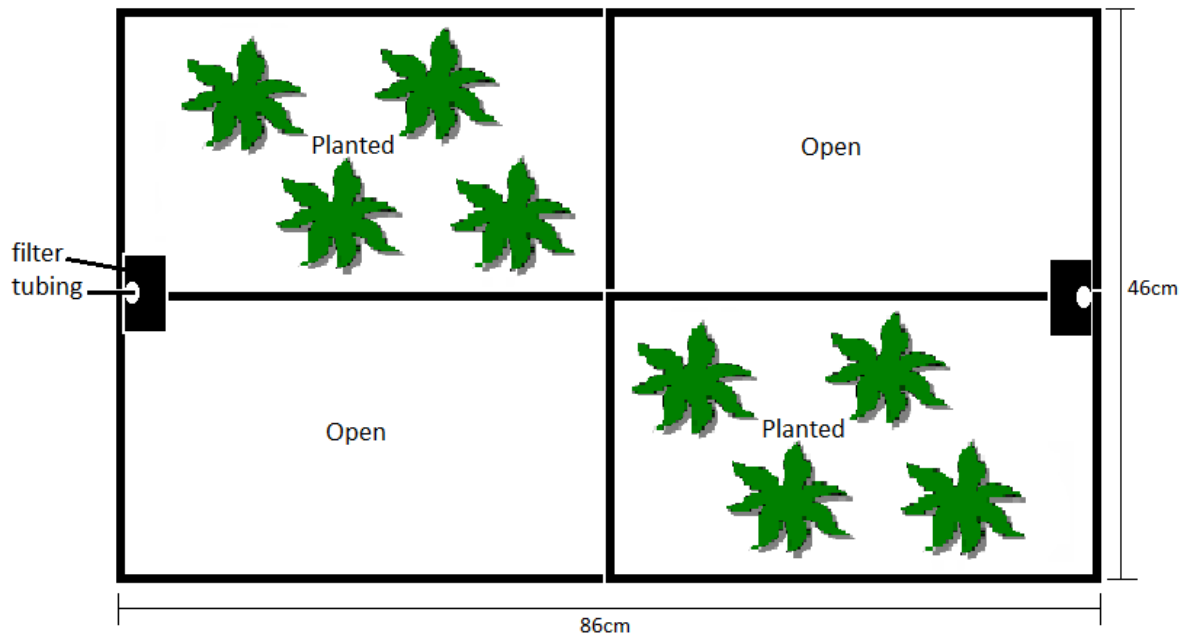


FIG. 4.2. Diagram of the experimental tank used in the area use predator scent test. Chemical cues were delivered down the tubing and dispersed by the pump. The water depth was maintained at 32cm deep.

4.3.6.3 Schooling following exposure to a live predator

To measure schooling in response to a live predator, the school was placed in an inflatable pool with a diameter of 105cm, filled to a level of 20cm and furnished with a small glass tank in the centre (46cm x 24cm) which was filled to the same level. The pool was light in colour with a pale blue base and no substrate was added, enabling the researchers to easily identify the fingerlings. The room was lit overhead by fluorescent lights and maintained at room temperature. The fingerlings were placed in the pool to settle prior to the beginning of the recording. After the settling period, the behaviour was recorded by an overhead camera. After the initial recording period, a single *L. unicolor* was added to the small tank in the centre of the pool and the behaviour of the fingerlings recorded on an overhead camera.

Data was collected by taking frame shots every 15s before and after the predator was added. TPS dig program (Rohlf 2013) was then used to create X,Y coordinates for the location of each fish. Based on this data and with reference to a scale, the inter-individual distance (IID) and the average distance of all individuals from the centre of the school (F-C) were calculated.

4.3.6.4 Schooling following exposure to predator scent

To measure schooling in response to predator scent the school of four fish was placed in an inflatable pool (diameter 85cm), with a water depth of 20cm. The pool was light in colour with a white base and no substrate, enabling the researchers to easily identify the fingerlings. The room was lit overhead with fluorescent lights and maintained at room temperature. In the centre of the pool, a piece of tubing was glued to the floor to allow the researcher to add predator scent (see 'treatments' for definition) without disturbing the fish. A small pump (capacity 150l/hr) was placed next to the end of the tubing to promote circulation of the scent throughout the pool.

The fingerlings were then placed in the pool, allowed to settle, and their behaviour recorded before and after the addition of the predator scent. The pool was drained completely after each test, cleaned and re filled with de-chlorinated water.

Data was collected by taking frame shots from the video footage every 15s before and after the predator cue was added. TPS dig program (Rohlf 2013) was then used to create X,Y coordinates for the location of each fish. Based on this data and with reference to a scale, the IID and F-C then were calculated.

4.3.7 Data Analysis

In the area use live predator test, the near zone use data was square root transformed and the open zone use data were log transformed to achieve normality. The data for planted zone use and for activity were normal and were left untransformed. For the area use predator scent test, area use data was transformed by a power of 4 and the activity data was log transformed to achieve normality. The schooling data was log transformed for the IID while the F-C data was normally distributed and was thus did not require transformation. Data was analysed using ANOVA and repeated measures ANOVA. Data were further scrutinized using a series of post-hoc tests (pairwise comparisons and one-way ANOVA) as required. All data analysis was conducted using Statview 5.0.1(SAS Institute Inc).

4.4 Results

4.4.1 Area Use tests

4.4.1.1 Area Use following exposure to a live predator

The use of the planted, open and near zone by conditioned fingerlings was investigated using a Repeated measures ANOVA. The results indicated that there was no treatment effect for zone use (planted; $F_{3, 44} = 1.33$, $P = 0.277$, open; $F_{3, 44} = 1.92$, $P = 0.140$, near; $F_{3, 44} = 0.68$, $P = 0.567$) nor was any time effect found for zone use by conditioned fingerlings (planted ; $F_{1, 44} = 1.95$, $P = 0.170$, open; $F_{1, 44} = 2.51$, $P = 0.120$, near; $F_{1, 44} = 0.69$, $P = 0.410$). No treatment- time interaction was found on area use by fingerlings in any of the three zones; planted ($F_{3, 44} = 1.41$, $P = 0.253$, Fig 4.3), open ($F_{3, 44} = 1.44$, $P = 0.243$, Fig 4.4) or near ($F_{3, 44} = 0.63$, $P = 0.597$, Fig 4.5).

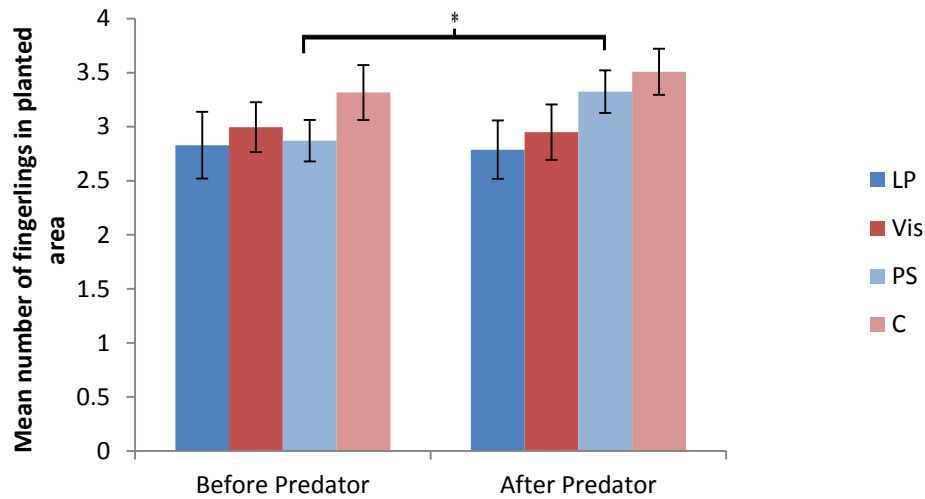


FIG 4.3. Mean (\pm SE) number of Australian bass fingerlings (from a total of 4) using the planted area during the live predator test in the 5 min intervals before and after predator exposure. Fingerlings were conditioned with one of four treatment; live predator (LP), predator scent (PS), visual cues (Vis) and control (C). (*indicates $P < 0.05$)

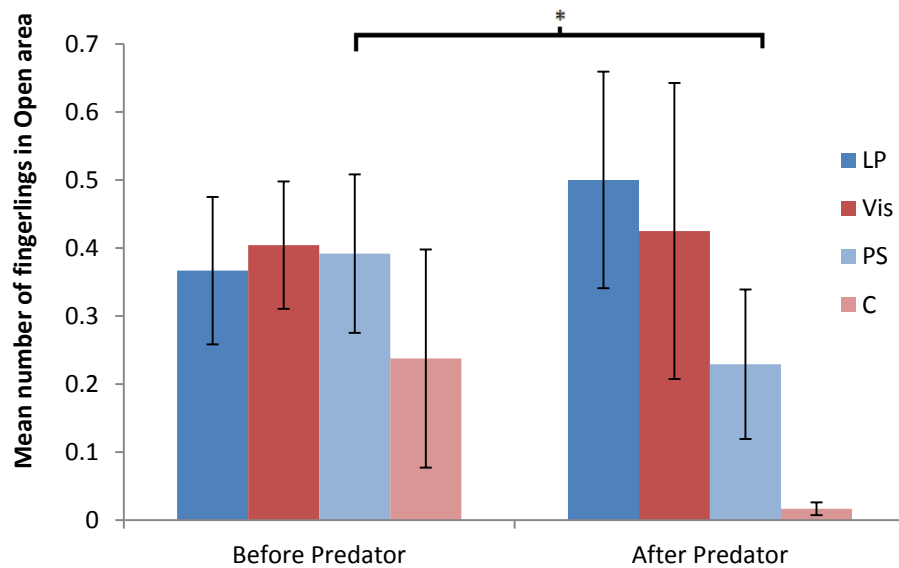


FIG 4.4. Mean (\pm SE) number of Australian bass fingerlings (from a total of 4) using the open area during the live predator test, in 5 min intervals. Fingerlings were conditioned with one of four treatment; live predator (LP), predator scent (PS), visual cues (Vis) and control (C).

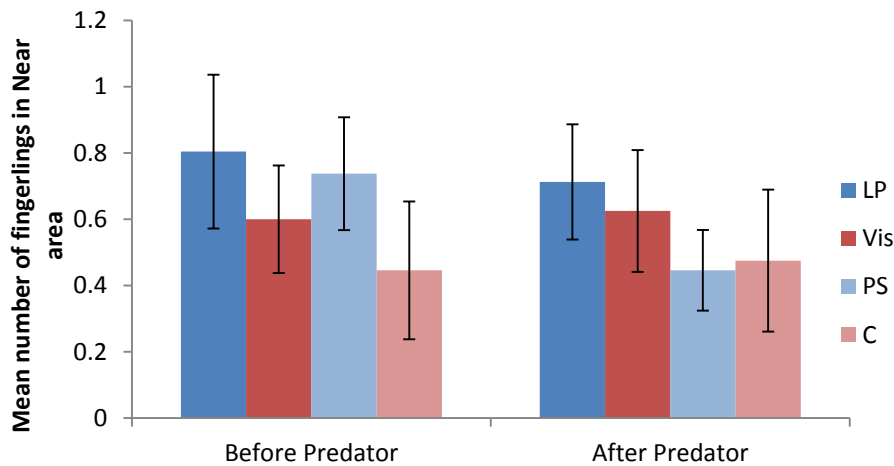


FIG 4.5. Mean (\pm SE) number of Australian bass fingerlings (from a total of 4) using the near area during the live predator test, in 5 min intervals. Fingerlings were conditioned with one of four treatments; live predator (LP), predator scent (PS), visual cues (Vis) and control (C).

Post-hoc analysis revealed that fish in the control treatment did not significantly change their zone use after the predator was revealed in either the planted, open or near zones. However, fingerlings from the predator scent treatment significantly increased their use of the planted zone after the predator was revealed ($F_{1,11} = 8.0$, $P = 0.017$, Fig 4.3) and subsequently decreased their use of the open zone ($F_{1,11} = 9.6$, $P = 0.010$, Fig 4.4). Pairwise comparisons indicated the live predator treatment increased their use of the open zone immediately after exposure to the predator (Fig 4.4), while the predator scent treatment decreased use of the open zone ($F_{1,22} = 4.48$, $P = 0.046$). A corresponding increase in use of the planted zone by the predator scent treatment in a pairwise comparison with the live predator treatment was also observed, but this failed to reach statistical significance ($F_{1,22} = 3.9$, $P = 0.062$). Neither treatment was statistically different from the control treatment.

Activity levels of conditioned fingerlings was also measured using a Repeated measures ANOVA and no treatment effect was found for activity ($F_{3, 88} = 1.80, P = 0.162$), however, there was an effect of time found for activity by conditioned fingerlings ($F_{2, 88} = 3.49, P = 0.035$; Fig 4.6) with no consistent pattern between treatments. No interaction between treatment and time was evident following exposure to a predator ($F_{6, 88} = 1.95, P = 0.082$). Post-hoc analysis revealed that fish in the control treatment showed no significant change in activity over time. However, fish in the predator scent treatment decreased activity after the introduction of a predator ($F_{2, 22} = 4.52, P = 0.023$). Pairwise analyses revealed the fish from the predator scent treatment were less active than those in the live predator treatment after the introduction of a predator ($F_{2, 44} = 5.60, P = 0.007$). Fish in the predator scent treatment were also initially more active than the control treatment fish but reduced activity after the introduction of a predator ($F_{2, 44} = 3.57, P = 0.037$). A decrease in activity was also observed between the fish from the predator scent treatment and the visual cues treatment ($F_{2, 44} = 3.10, P = 0.055$) but this failed to reach statistical significance.

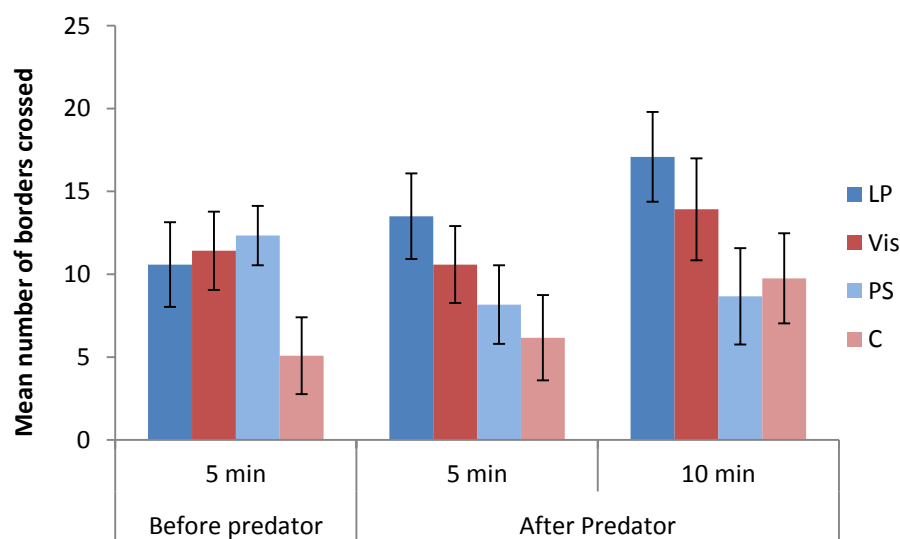


FIG 4.6. Mean (\pm SE) number of border crossings (activity) of Australian bass fingerlings during the live predator test 5 min before and 5 and 10 min after exposure to a predator. Fingerlings were conditioned with one of four treatments; live predator (LP), predator scent (PS), visual cues (Vis) and control (C).

4.4.1.2 Area use following exposure to predator scent

Behaviour of conditioned fingerlings was examined using the final 5 min of observation before the predator cue was added, and the ensuing 20 min after the cue was added. The results of a repeated measures ANOVA indicate there was no main effect of treatment on cover use (planted zone) ($F_{3, 176} = 0.98, P = 0.413$, Fig 4.7); however, a strong effect of time was evident ($F_{4, 176} = 12.34, P = <0.0001$) with an increase in cover use observed immediately after the predator was revealed. There was also no treatment by time interaction on use of cover after conditioned fingerlings were exposed to predator scent ($F_{12, 176} = 1.68, P = 0.075$). Further to this, the final 5 min interval of the 'before' period was compared with the initial 5 min interval after the predator scent was added, and a significant interaction of treatment by time was observed ($F_{3, 44} = 3.74, P = 0.018$). There was no main effect of treatment ($F_{3, 44} = 0.82, P = 0.491$); however, a strong effect of time was also evident here ($F_{1, 44} = 28.29, P = <0.0001$). Further investigation of these two time intervals, 5 min prior to predator exposure and 5 min after predator exposure, showed the fish in both the control and the live predator treatments increased use of cover significantly after the predator scent was added ($F_{1,11} = 11.15, P = 0.007, F_{1,11} = 29.70, P = 0.0002$ respectively). Pairwise post hoc tests indicated the fish from the live predator treatment made less use of cover, particularly prior to the addition of the predator cue, than fish in the predator scent ($F_{1, 22} = 10.68, P = 0.004$) and the visual cues treatments ($F_{1, 22} = 4.34, P = 0.049$). However, neither of these treatments was significantly different from the control treatment.

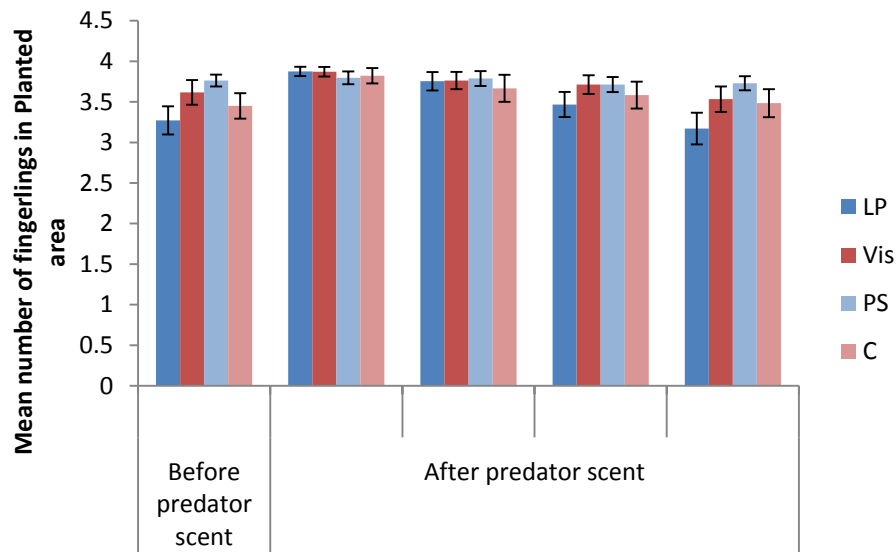


FIG 4.7. Mean (\pm SE) number of Australian bass fingerlings (from a total of 4) using the planted area during the predator scent test, in 5 minute intervals. Fingerlings were conditioned with one of four treatment; live predator (LP), predator scent (PS), visual cues (Vis) and control (C).

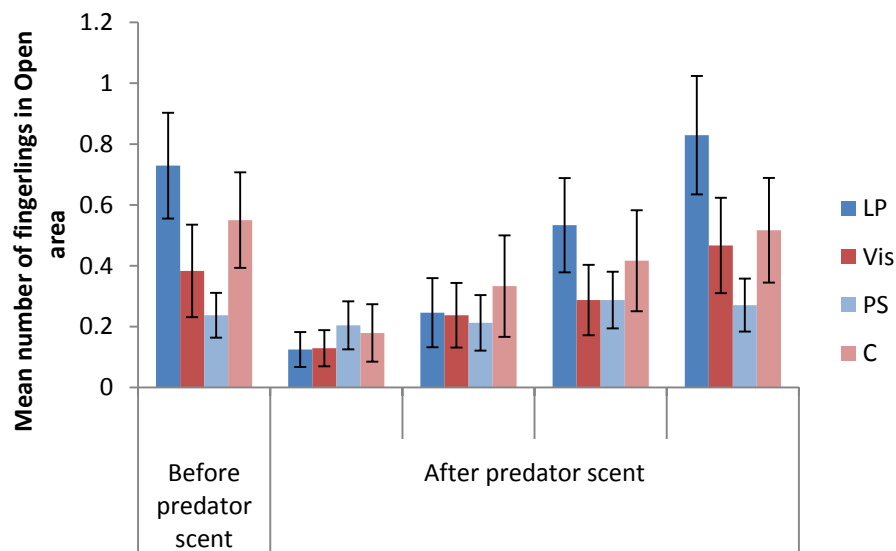


FIG 4.8. Mean (\pm SE) number of Australian bass fingerlings (from a total of 4) using the open area during the predator scent test, in 5 min intervals. Fingerlings were conditioned with one of four treatment; live predator (LP), predator scent (PS), visual cues (Vis) and control (C).

There was no main effect of treatment evident for activity levels in conditioned fingerlings exposed to predator scent ($F_{3, 176} = 1.01$, $P = 0.396$, Fig 4.9); however, a strong effect of time was evident ($F_{4, 176} = 11.18$, $P = <0.0001$). There was also no treatment - time interaction for activity levels in conditioned fingerlings ($F_{12, 176} = 0.60$, $P = 0.841$). Post hoc tests revealed all treatments showed a change in behaviour over time, with an initial drop in activity after the predator cue had been added (control; $F_{4, 44} = 3.87$, $P = 0.009$, live predator; $F_{4, 44} = 5.07$, $P = 0.002$, visual cues; $F_{4, 44} = 3.84$, $P = 0.009$), with the exception of the predator scent treatment ($F_{4, 44} = 0.73$, $P = 0.579$) where activity levels stayed more stable. This initial drop was followed by a gradual recovery. However, pairwise post hoc tests indicated no treatment behaved significantly differently from any others.

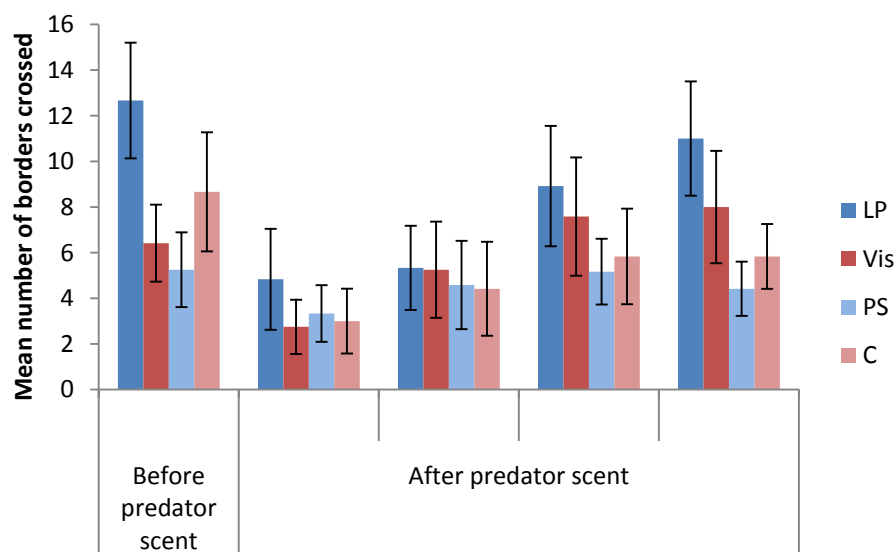


FIG 4.9. Mean (\pm SE) number of border crossings (Activity) of Australian bass fingerlings during the Predator Scent test, in 5 min intervals. Fingerlings were conditioned with one of four treatments; live predator (LP), predator scent (PS), visual cues (Vis) and control (C).

4.4.2 Schooling tests

4.4.2.1 Schooling following exposure to a live predator

Data was analysed using the final 5 min prior to and the 0-5 and 5-10 min intervals after the introduction of a predator in a Repeated Measures ANOVA. No main effect of treatment was found, measured as either Inter-individual Distance (IID) ($F_{3, 88} = 0.68$, $P = 0.568$, Fig 4.10) or average distance of a fish from the centre of the school (F-C) ($F_{3, 88} = 0.35$, $P = 0.791$, Fig 4.11); however, an effect of time was evident in both cases (IID; $F_{2, 88} = 4.17$, $P = 0.019$, F-C; $F_{2, 88} = 237.57$, $P = <0.0001$) with a general increase in IID and F-C observed immediately after exposure to the predator indicative of reduced schooling. In addition to this, no treatment-time interaction effect on schooling behaviour was observed for IID ($F_{6, 88} = 1.37$, $P = 0.236$) or F-C ($F_{6, 88} = 0.90$, $P = 0.499$). Post hoc tests of the IID show the schooling behaviour of the fish in the control treatment remained the same after the predator was added. Fish in the predator scent treatment, however, exhibited an initial increase in IID after the predator had been introduced ($F_{2, 22} = 4.74$, $P = 0.019$). Further pairwise comparisons indicated the predator scent treatment fish showed a tendency towards a greater IID than did the live predator treatment fish ($F_{2, 44} = 3.10$, $P = 0.055$), however this failed to reach statistical significance and neither treatment was statistically different from the control treatment. Post hoc analysis of the F-C data showed all treatments responded strongly to the addition of a predator ($P < 0.0001$ in all cases) with a large increase in F-C distance, however, no treatment responded significantly differently from any other.

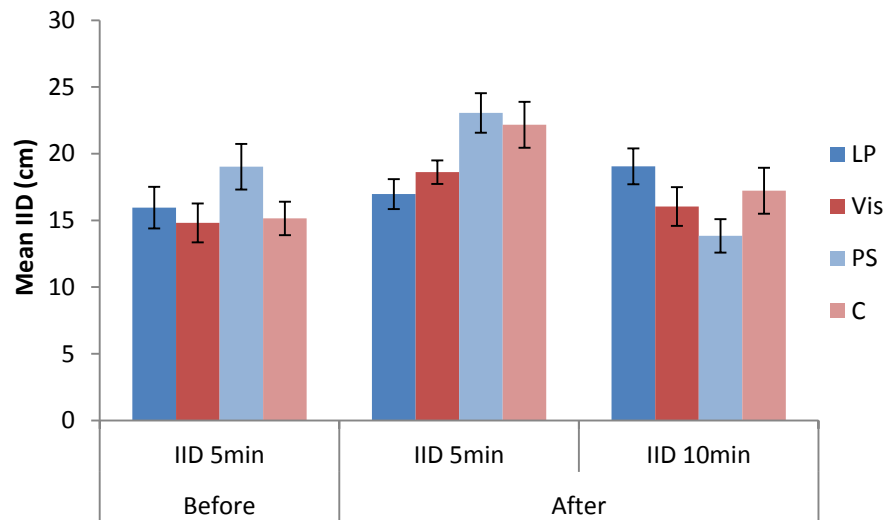


FIG. 4.10. Mean (in cm, \pm SE) Inter-individual Distance (IID) observed in 5 min intervals before and after a predator was introduced. Fingerlings were conditioned with one of four treatment; live predator (LP), predator scent (PS), visual cues (Vis) and control (C).

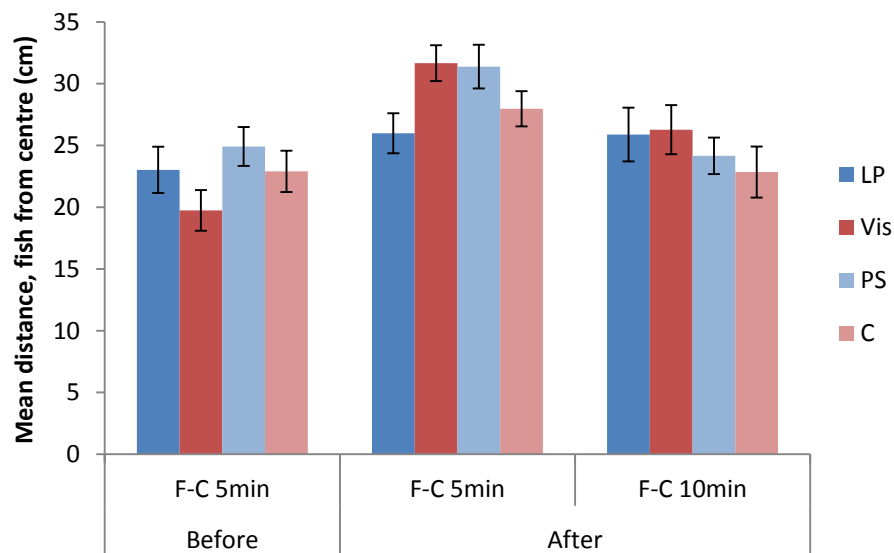


FIG. 4.11. Mean (in cm, \pm SE) distance of a fingerling from the centre of school (F-C) observed in 5 minute intervals before and after a predator was introduced. Fingerlings were conditioned with one of four treatment; live predator (LP), predator scent (PS), visual cues (Vis) and control (C).

4.4.2.2 Schooling following exposure to predator scent

There was no main effect of treatment found in either Inter-individual Distance (IID) ($F_{3, 88} = 0.04$, $P = 0.991$, Fig 4.12) or average distance of a fish from the centre of the school (F-C) ($F_{3, 88} = 0.31$, $P = 0.818$, Fig 4.13) nor was an effect of time evident (IID; $F_{2, 88} = 0.09$, $P = 0.914$, F-C; $F_{2, 88} = 0.58$, $P = 0.563$). In addition to this, no interaction between treatment and time was observed, measured as either IID ($F_{6, 88} = 0.95$, $P = 0.462$) or F-C ($F_{6, 88} = 0.79$, $P = 0.582$) in conditioned fingerlings. Post hoc tests revealed no significant interactions over time or between treatments. A trend towards increased schooling was observed in the visual cues treatment, however it did not achieve statistical significance.

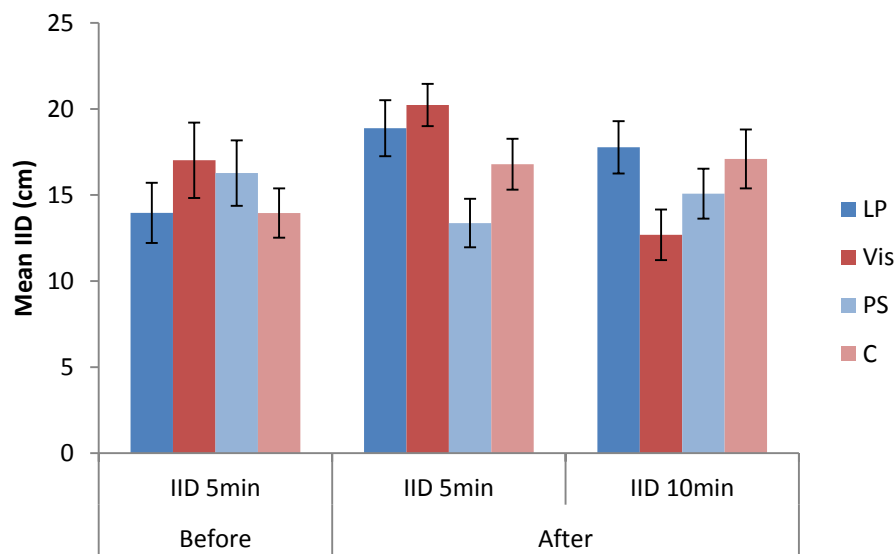


FIG. 4.12 Mean (in cm, \pm SE) Inter-individual Distance (IID), observed in 5 min intervals before and after predator scent was added to the water. Fingerlings were conditioned with one of four treatments; live predator (LP), predator scent (PS), visual cues (Vis) and control (C).

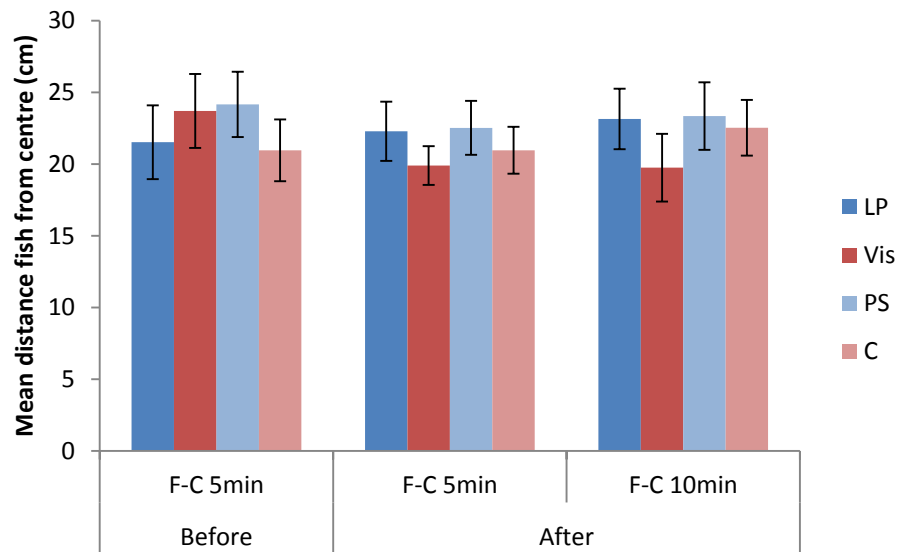


FIG. 4.13 Mean (in cm, \pm SE) distance of a fingerling from the centre of school (F-C) observed in 5 min intervals before and after predator scent was added to the water. Fingerlings were conditioned with one of four treatment; live predator (LP), predator scent (PS), visual cues (Vis) and control (C).

4.5 Discussion

4.5.1 Area use tests

In this study, two main alterations in the behaviour of conditioned juvenile *M. novemaculeata* were observed; those present after the addition of predator cues and those evident both before and after the predator cues that differ from the control. These changes appeared to also be dependent on the test and the treatment used. In general, the changes in behaviour observed were the result of present information about the predator and prior learning experiences. Differences between treatments appear to be graded, suggesting a flexible approach to anti-predator behaviour that is context specific and threat sensitive in nature.

When the fingerlings were tested for area use in the presence of a live predator, the fingerlings conditioned with a live predator tended to spend more time in the zone closest to the predator, both before and after the predator was revealed. Conversely, the fingerlings from the predator scent treatment increased their use of cover after the predator was revealed, spending more time in the planted zone, and less time in the open zone. After the predator was revealed, fingerlings from the live predator treatment spent more time in the open zone than the fingerlings from the predator scent treatment. If the fingerlings from the live predator treatment were participating in predator inspection behaviour it would seem intuitive that they would increase use of the zone near the predator. Whilst they did not increase use of cover, they did increase distance from the predator. Likewise the fingerlings conditioned using predator scent reduced activity much more than those conditioned using a live predator. It seems the fingerlings from the predator scent treatment were much more inclined to react timidly, while the live predator treatment was not so sensitive to the appearance of the predator. It is possible the difference between treatments observed here is the fish responding in a threat sensitive fashion, where the responses are graded according to the threat levels the fingerlings were conditioned with.

Helfman (1989) proposed the threat-sensitivity hypothesis in a study where damselfish *Stegastes planifrons* (Cuvier 1830) were exposed to progressively greater predator threat and matched their avoidance behaviour accordingly. Likewise, Ferrari *et al* (2005) conditioned minnows *Pimephales promelas* (Rafinesque 1820) to recognise a predator using varied concentrations of alarm cue. In later recognition trials, the intensity of anti-predator response was retained, showing a learned response to high risk predation cues. Conversely, high background threat levels may contribute to a lowered anti-predator response. Brown *et al* (2006) showed cichlids *Amatitlania nigrofasciata* (Günther 1867) exposed to high background levels of risk had lower intensity responses to alarm cues, as is predicted by the risk allocation hypothesis. This may account for the less intense reaction exhibited by the fingerlings from the live predator treatment when compared with those from the predator scent treatment.

The fingerlings subjected to visual cues during training behaved in an intermediate fashion and showed no significant changes in area use or activity. This is interesting and may be explained by a species preference to learn using multiple or chemical cues. Indeed, a number of studies have noted a combined or additive effect to anti-predator response when prey are presented with multiple cues (Smith and Belk 2001; McCormick and Manassa 2008; Kim et al. 2009; Manassa et al. 2013). This may also be context specific and reflect the environmental conditions preferred by the species. For example, larval newts *Notophthalmus viridescens* (Rafinesque 1820) show better discrimination of predators using chemical cues rather than visual cues which is likely a consequence of the environmental conditions which compromise visibility (Mathis and Vincent 2000). Australian bass *M. novemaculeata* prefer habitats with dense aquatic vegetation (Allen et al. 2002) and thus are likely to rely more heavily on chemical cues in those situations. Chemical cues are easily dispersed and available in the aquatic environment and are widely used in learned predator recognition (Brown and Chivers 2007; Ferrari et al. 2010). Furthermore, visual cues are unlikely to occur without corresponding olfactory cues which may account for the modest response to learning from visual cues only.

When the fingerlings were tested for area use in the presence of predator scent, the difference in responses was similar to that observed when they were tested with a live predator, that is to say, the fingerlings from the predator scent treatment showed the more intense response. In the first 5min after the predator scent had been added to the tank, a ceiling value was seen across all treatments. Possibly this was due to the water being added, although all endeavours were made to avoid this being disruptive. In both area use and activity, fingerlings from the predator scent treatment were slowest to recover, indicating again, a more intense response from fingerlings trained using olfactory cues only. The fingerlings from the predator scent treatment appeared to already be exhibiting increased anti-predator behaviours (such as increased use of cover and reduced activity) prior to the addition of a predator cue, whilst the fish in the control and Live

Predator treatments showed less intense responses before the addition of predator cue and recovered faster after the addition of predator cue.

The prior experience of the fingerlings (ie their conditioning) resulted in a graded response across the treatments. Experience with predators can vastly alter the anti-predator behaviour of prey. Fish from populations experiencing high predation are better at avoiding predators (Magurran and Seghers 1990; Brown and Warburton 1999). While some of this response is inherited, there is also a significant learning component. Indeed, Berejikian (1995) was able to enhance the ability of hatchery-reared trout *O. mykiss* to avoid predation after they had experience with a predator. In this study, previous experience with predator cues appears to elicit a generalised increase in anti-predator behaviour relative to threat levels during conditioning. The responses, however, were somewhat different and less intense in the predator scent test. This is likely due to a lower perceived level of risk, resulting from the absence of associated visual cues. In gobies *A. semipunctatus*, the addition of visual information resulted in a different set of behaviours being elicited than those seen using just olfactory cues (McCormick and Manassa 2008). Macquarie perch *Macquaria australasica* (Cuvier 1830) exposed to chemical cues alone showed no change in the position in the water column but reduced their activity levels. In the presence of visual cues, however, they participate in predator inspection whilst generally maintaining a safe distance from the predator (Morgan & Brown 2013). When visual cues are absent, the fingerlings may have to generalise their response and tailor it to the present level of risk. These results suggest a complex interaction between previous experience and background threat levels (conditioning) and perceived immediate threat (predator cue during test). This highlights the plastic nature of predator prey interactions and invites further investigation in the area.

4.5.2 Schooling tests

When schooling was tested using a live predator, fish in all treatments changed their behaviour in response to the addition of a predator in the pool. The disruption of a predator being placed in the

experimental arena will undoubtedly have contributed to the initial response, however, differences between treatments were also observed. The fingerlings from the predator scent treatment exhibited a trend towards looser schooling behaviour before the introduction of a predator, then an initial increase in IID upon the addition of a predator, followed by a recovery period where IID decreased. Magurran and Pitcher (1987) showed that after the detection of a predator minnows *Phoxinus phoxinus* (L. 1758), formed a single compact school, after which they began predator inspections. It is possible the *M. novemaculeata* fingerlings from the predator scent treatment were undertaking predator inspection, thus accounting for the reduced schooling behaviour immediately after the predator was added to the arena. Predator inspection is a risky behaviour, however it does provide valuable information about the threat posed by a predator (Kelley and Magurran 2007). Predator inspection has a role in risk assessment, and can help the prey gain information that is immediately relevant to the threat posed by a predator (Magurran and Pitcher 1987). Furthermore, prior experience with predators has been shown to elicit increased predator inspections in *P. phoxinus* (Magurran 1990). In this study, experience with predators is, once more, likely to have impacted on the behavioural choices the fingerlings made. The fingerlings from the live predator treatment and the visual cues treatment showed fairly stable trends in schooling behaviour and recovered quickly. Interestingly the fingerlings from the predator scent treatment behaved in a way that was similar to the control fish, showing looser schooling behaviour, yet making a larger recovery. It is possible the fish in these treatments behaved this way as they were unaccustomed to the visual presence of a predator whereas fingerling from the visual cues and live predator treatments had prior experience with visual predator cues. Chapman *et al* (2010) examined the behaviour of *P. reticulata* reared in low light conditions. They found the *P. reticulata* responded with sensory plasticity, where the fish reared in low light performed better in tests using the cues they were experienced in during rearing (i.e. olfactory).

When tested using a predator scent cue, a different set of responses were observed. While there were trends towards tighter schooling by the fingerlings in the predator scent and control

treatments nothing was statistically significant. This in itself is interesting and suggests the fingerlings felt less of an immediate threat from olfactory cues alone. Olfactory cues warn of a predator in the vicinity whereas visually observing a predator implies an immediate threat (Brown and Chivers 2007). The threat of predation was insufficient to elicit a dramatic change in their schooling behaviour. Brown *et al* (2004) found when shoals of glow-light tetras *Hemigrammus erythrozonus* (Durbin 1909) were exposed to sub threshold levels of alarm cue, they showed little anti-predator response, yet the sight of an alarmed conspecific was sufficient to increase their anti-predator response. This begs the question, if further cues were given, would the conditioned fingerlings respond in a way that was similar to their reaction during the schooling after exposure to a live predator test? Whilst no conclusive interactions can be drawn from this test, the results of the other tests do show that both visual and chemical cues play a role in learned predator response in *M. novemaculeata*.

M. novemaculeata are a popular target species for recreational fishing and have a long stocking history in Australia. Their range is extensive and stretches from the Mary River to Wilson's Promontory along the South-East coast of Australia, where they favour areas of aquatic vegetation (Allen et al. 2002). Given the vast number stocked and popularity of stocking *M. novemaculeata*, it would be prudent to make efforts to improve the success of released bass fingerlings. The use of pre-release training in hatchery reared fish has gained popularity over recent years, and many leaps have been made in our increased understanding of the topic (Olla et al. 1998; Brown and Laland 2001; Vilhunen 2005). By training the hatchery fish to recognise and avoid predators there is the potential to vastly improve survival rates of released fish. Recently, efforts have been made to demonstrate predator recognition using olfactory cues can be achieved on a larger scale with success (Olson et al. 2012). Further to this, others have shown conditioning hatchery-reared fish results in a vastly improved survival post-release (D'Anna et al. 2012). Studies of this sort focusing on Australian species are still in their infancy, however, work done by researchers for the Murray-Darling Basin Authority (Hutchison *et al.* 2012) shows encouraging results both in laboratory trials

and in the field. The results presented in this study emphasise the complex and plastic nature of learned predator recognition, and highlight the need for a species-specific tailored approach to life skills training. These results also suggest pre-release conditioning using both visual and olfactory cues from a predator could potentially improve the anti-predator response of this species post release. Clearly the different sensory modes play different roles in learning and further research remains to be done to fully understand and utilise this information.

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Chapter 5

Behavioural interactions between conditioned, hatchery-reared Australian

Bass *Macquaria novemaculeata* and predatory spangled perch

***Leiopotherapon unicolor* in a semi-natural enclosure**

This chapter is formatted for publication in the Journal of Fish Biology. All figures and tables are embedded in text.

5.1 Abstract

The hatchery environment is known to hinder a complete development of anti-predator behaviours in fish. This is largely due to environmental differences between the hatchery and natural environments, most notably, a lack of experience with predators. Life-skills training aims to overcome this by conditioning hatchery reared fish to recognise predators and improve anti-predator skills. Here we condition hatchery reared Australian bass *Macquaria novemaculeata* fingerlings using both visual and olfactory cues from a predatory spangled perch (*Leiopotherapon unicolor*) paired with damage-released conspecific extract. The fingerlings were then tested in a trial in semi-natural conditions with a predator at liberty. The results show conditioning changes the behaviour of the bass fingerlings, with a combination of visual and olfactory cues invoking the greatest change. A graded response was seen in the other treatments, with those containing a visual element exhibiting a stronger response. These results show the potential for pre-release training to be used in this species with a view to improving post release survival of hatchery reared juveniles.

Keywords: Australian bass, *Macquaria novemaculeata*, hatchery rearing, predator recognition, life-skills training.

5.2 Introduction

The ability to identify predators and respond appropriately is an important skill required by prey animals. The development of many key behaviours often involves learning through experience early in development, thus rearing fish in artificial environments can create numerous behavioural deficits. The ability to recognise and respond appropriately to predators is one of the key ways in which hatchery reared fish differ when compared to their wild counterparts. Lack of experience with predators in the hatchery environment is considered one of the main factors that contribute to this under-development of behavioural skills (Olla *et al.*, 1998). By facilitating interactions between hatchery reared fish and predator cues, life skills training aims to overcome some of these behavioural shortcomings (Brown and Laland, 2001).

When identifying a predator and assessing risk, prey fish may use a range of cues. The assortment of attributes unique to each of the different sensory modalities makes their utility in certain contexts distinct. Visually acquired data is temporally more reliable, yet the proximity required to visually assess a predator means that gaining visual information about predators is inherently risky. On the other hand, olfactory cues travel easily through the aquatic environment and can function as an early warning (Chivers *et al.*, 2001). If one sensory modality is unreliable or unavailable, an increased use of others may occur. An example of such sensory compensation was observed in Atlantic salmon, that make greater use of olfactory cues at night than they do during the day (Leduc *et al.*, 2010). Furthermore, cues may be used differentially during different tasks. For instance, mosquitofish *Gambusia affinis* (Baird & Girard, 1853) rely more on visual cues than on chemical cues during risky behaviours such as predator inspection (Smith and Belk, 2001).

Despite the range of predator cues available to prey fish, the type and intensity of the response issued by prey often follows a threat sensitive pattern. All prey animals must trade-off time spent being vigilant with other major activities such as foraging or finding mates. The threat sensitive hypothesis predicts that the intensity of the response exhibited by prey is dependent on the

perceived level of threat posed by predators (Helfman, 1989) and a number of studies have investigated the response to predator cues in a threat sensitivity context. Thus the manner in which prey respond to different cues is likely to relate to the level of threat perceived from the cue, with cues signifying high threat levels eliciting the most intense anti-predator response (McCormick and Manassa, 2008; Holmes and McCormick, 2011). In doing this, prey maximise fitness by avoiding unnecessary energy expenditure or lost foraging opportunities whilst partaking in anti-predator behaviour.

Recognition of predators has a large learned component and is often achieved by associating cues emanating from damaged conspecifics with cues from the predator. The use of associative learning in predator recognition allows the prey fish to tailor their responses to the local conditions. Fish reared in hatcheries are generally denied experience with predators and thus lack the learned skills necessary for survival (Olla *et al.*, 1998). Recently, however, increasing amounts of interest have gone into research focusing on behavioural rehabilitation of naive hatchery-reared fish prior to their release, usually involving predator conditioning (Brown and Laland, 2001).

Pre-release training aims to overcome some of the behavioural issues experienced as a consequence of hatchery rearing. In a predation context, this is often done by conditioning hatchery reared fingerlings to associate the odour of a predator with damaged released conspecific alarm pheromones. The use of such predator conditioning has been widely studied and an increasing body of evidence shows it is an effective method of improving anti-predator skills in predator naïve fish. After just a single conditioning event, hatchery reared fish can significantly improve their ability to recognise and respond to a predator (Brown and Smith, 1998). Mirza and Chivers (2000) demonstrated that conditioning improved the survival of juvenile brook trout *Salvelinus fontinalis* (Mitchill 1814) in both laboratory and field enclosure encounters with a predator. Likewise, an improvement in post release survival was observed in hatchery-reared white seabream *Diplodus*

sargus (L. 1758) by providing experience with shelters and predator conditioning (D'Anna *et al.*, 2012).

More recently, studies have investigated the use of pre-release training on survival in large groups of fish, demonstrating it was possible to condition large numbers of hatchery reared fish in an economical fashion (Olson *et al.*, 2012). Furthermore, researchers for the Murray-Darling Basin Authority (MDBA) have investigated the technique as a possible process for improving the post-release survival of some commonly stocked species of Australian fish (Hutchison *et al.*, 2012). Their findings validated that predator conditioning could improve the anti-predator behaviour of three species of Australian freshwater fish, silver perch *Bidyanus bidyanus* (Mitchell 1838), catfish *Tandanus tandanus* (Mitchell 1838) and Murray cod *Muchullochella peelii* (Mitchell 1838). Not only was an improvement in behaviour observed, but a substantial increase in survival after release was seen in the *M. peelii*. This study highlighted the need for greater research outlining the benefits of pre-release training for those species that are commonly stocked in Australian waters.

Australian bass *Macquaria novemaculeata* (Steindachner 1866) are a large fresh water fish from eastern Australia. They are a popular target species for recreational fishermen and are produced in large numbers for stocking in farm dams and impoundments. The stocking plan for the 2012/2013 period by the NSW Department of Primary Industries estimated over 280,000 would be released into dams and lakes (DPI, 2012/2013). Despite the popularity of the species, little is known about the success of juvenile *M. novemaculeata* post-release. In addition, little is known about how they interact with predators. Here we examine the anti-predator behaviour of conditioned hatchery reared, juvenile *M. novemaculeata*. Firstly the use of cover and of discreet behaviours is investigated in a semi-natural setting in the presence of a predator at liberty. Secondly, the relative roles of different predator cues during the conditioning phase were examined. The aim of this paper was to gather information on predator conditioning in this species that may be of utility in improving stocking success.

5.3 Methods

5.3.1 Subjects and housing

Subjects were young of the year *M. novemaculeata* fingerlings, of around 5cm T_L, produced under the Hatchery Quality Assurance Scheme (HQAS) and reared in outdoor dams. The fingerlings that were used were the first generation offspring of wild caught broodstock. The fingerlings were transported overnight in oxygenated water, packed in Styrofoam boxes. Upon arrival at Macquarie University, Sydney, the fingerlings were transferred into outdoor ponds (capacity 1530L) at a density of 55 fish per pond. The ponds were maintained under bird netting and shade cloth to avoid avian predation. During the first week, the fingerlings were weaned onto flake food using bloodworms. They were fed flake food 5 days a week and maintained in these outdoor housing ponds until the beginning of experiments.

The fish were then moved into tanks within a laboratory for conditioning prior to testing. The conditioning tanks measured 90cm long x 35cm wide and were filled to 35cm deep. Tanks were placed flush with the wall and all other sides of the tanks (excluding the top) were covered to minimise visual interaction and prevent undue stress on the fish from the activity of lab users. The room had a 12hrs light: 12hrs dark light cycle. Each aquarium was fitted with a filter (capacity 500l/hr), gravel and heater set at 16°C. The fish were placed in these tanks the day before conditioning started, to allow them to settle.

Three wild caught, mature, spangled perch *Leiopotherapon unicolor* (Günther 1859) were used as predators as they have a reputation as voracious predators and have an extremely large distribution. The Spangled perch were housed individually in 60 l aquaria, lit overhead by fluorescent lights with a 12 hr light: 12hr dark light cycle. Each aquarium was fitted with a filter (capacity 500l/hr), gravel and heater set at 18°C. They were fed a diet of frozen prawns four times per week. Feeding was suspended during experimental periods to enhance hunting motivation. The predator's housing tank had no water changes and was not cleaned for a week prior to the conditioning stage. They were not

used for more than 2 sequential trials to maintain motivation. After the conditioning, they were placed back in their housing tank and their feeding resumed.

5.3.2 Conspecific extract (CE)

CE was generated by homogenising whole conspecifics and passing the liquid through a filter (see Chapter 2, Methods 2.3.3.1 for details).

5.3.3 Conditioning

A total of 15 replicate schools were tested per treatment. Fingerlings were tested in groups of three. The fingerlings were conditioned to predator cues in groups of 24 fingerlings per tank (i.e. 8 schools). Two batches of eight schools were conditioned per treatment to achieve a total of 16 replicate schools per treatment. This included an additional 3 fish per treatment (one school) that could be discarded from the testing if they became too stressed during the conditioning. Four separate tanks were used to condition the fingerlings. Each tank was designated to one of 4 treatments (see below). Each group of fingerlings was exposed 3 times per day to the predator stimuli between the hours of 9am – 10am, noon – 1pm and 3pm – 4pm for 3 days (a total of 9 exposures). Each exposure lasted 15min. This level of training was determined by a series of pilot studies. The four treatments were as follows;

5.3.3.1 Predator scent combined with CE

During this treatment (henceforth abbreviated to predator scent treatment) fingerlings received a combination of conspecific extract and predator scent only (i.e. visual cues excluded). Water that contained the chemical signatures of the predator was used as predator scent. Immediately prior to exposure, 20ml of water was collected from a 60l tank containing a single *L. unicolor* who had been housed in the tank (sensu Brown and Smith, 1998). The predator tank had no water changes or cleaning done for a week prior to the conditioning stage. The scent was added to the conditioning tank immediately followed by 20ml CE. At the completion of each conditioning the tank was drained

completely, cleaned and refilled with de-chlorinated tap water, ready for the next group to be conditioned.

5.3.3.2 Live predator combined with CE

During this treatment (henceforth abbreviated to live predator treatment) a live predator was placed behind a mesh barrier within the conditioning tank. A partition made from fine mesh (2mm²) was placed at 1/3 of the length of the tank along, creating two sections; a large section (measurements 62cm x 35cm depth x 35cm wide) and a small section (measurements 31cm x 35cm depth x 35cm wide). The predator was placed in the small section of the tank whilst the fingerlings were placed in the large section. Water was able to pass through the partition, allowing circulation of water and chemical cues. An opaque partition placed adjacent to the mesh was attached to a pulley and could be lifted remotely. The partition was lifted for a duration of 15min at each exposure, to allow visual contact with the predator. This was done in conjunction with the addition of 20ml CE. The predator was removed over night to prevent it attempting to escape. At the completion of each conditioning bout the tank was drained completely, cleaned and refilled with de-chlorinated tap water.

5.3.3.3 Visual cues only combined with CE

Fish in the visual cues only combined with CE (henceforth abbreviated to visual cues treatment) received a combination of CE and visual contact with the predator, in the absence of any chemical cues from the predator. Fingerlings were placed in a tank covered with black plastic on three sides to limit any other visual cues. On one short side, a tank of the same dimensions containing a predator was placed flush with the fingerling tank. An opaque Perspex barrier was placed between the two tanks and was attached to a remote pulley that allowed it to be lifted, providing visual contact between the two tanks. At the conditioning times, the predator was gently guided to the end of its tank closest to the fingerlings and contained there by placement of a clear barrier at 30cm away from the end of the tank. The visual barrier between the two tanks was lifted and the CE carefully

added to the fingerlings tank at the end nearest the predator. The barrier remained up for 15min. At the end of the 15min, it was lowered and the predator was released by removing the clear partition. At the completion of each conditioning bout the fingerlings tank was drained completely, cleaned and refilled with de-chlorinated tap water.

5.3.3.4 Control

This treatment was representative of standard hatchery fish used for stocking and therefore received no conditioning. The fingerlings were placed in conditioning tanks of the same dimensions and water volume as the other treatments the day prior to when conditioning would begin. In place of the exposures, the control group were simply left in the aquaria for the equivalent amount of time. The tank was drained completely, cleaned and refilled with de-chlorinated tap water before the next group of fish was conditioned.

5.3.4 Experimental Procedure

The testing was done using a glass tank measuring 245cm x 48cm deep x 61cm tall, this was divided into 2 by a barrier placed 60cm from one end to create a predator zone, where the predator could be released into the testing area with the fingerlings (Fig. 5.1). The testing area was marked at every 30cm to indicate the different 'zones'. Zones alternated between open and planted from 1 to 6 up to the predator zone. Planted zones were furnished with plants and hides to provide extensive cover for the fingerlings to seek refuge in. Each planted zone contained 1 black, round pot (140mm diameter) with the base cut out and weights glued to the side to sink it, creating a cylinder large enough for the predator to swim through. Each planted zone also contained a small, black, square punnet (50mm at widest), similarly adjusted, that the predator was too large to comfortably fit into. In addition, each planted zone contained 5 large plastic aquarium plants. This combination of items gave dense cover and ample hiding places. The orientation of each of these items within their planted zone was changed between each trial, so the predator did not become too familiar with the

layout. Open zones were left clear of plants and other items. A thin layer of pebbles covered the entire tank to reduce the reflectiveness of the glass base.

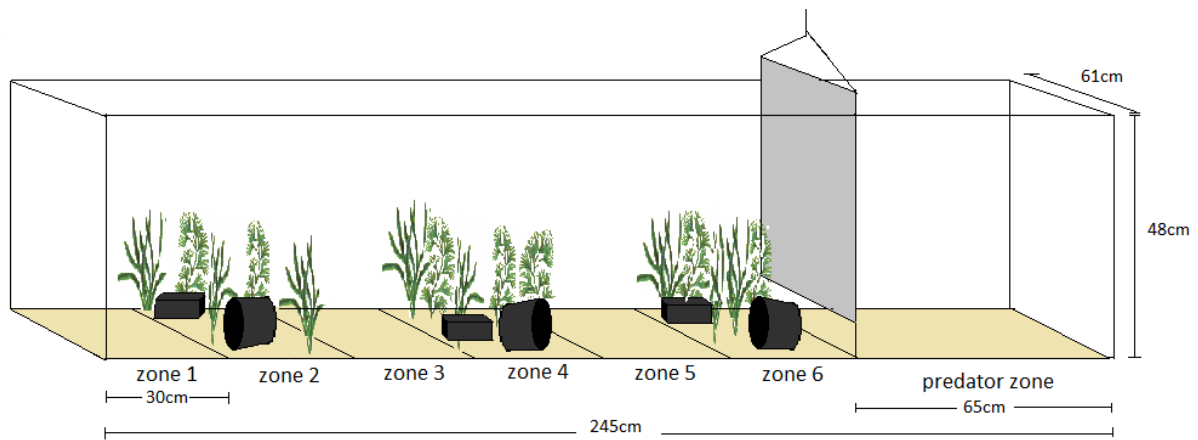


FIG. 5.1. Layout of the experimental tank. Zones 1, 3 and 5 were 'open' zones with no structures and zones 2, 4 and 6 were 'planted' zones, furnished with plants and refuges. The predator zone was separated by a Perspex barrier that could be lifted remotely.

Fingerlings were tested in groups of three. They were placed in the testing tank and given 1 hour to settle, before beginning a 20min test. The behaviour of both fingerlings and predator was observed. The use of refuge and open areas (termed area use), activity (measured by counting borders crossed), latency to first move and distinct behaviours were recorded. Fingerling behaviours recorded included startle, approach, escape and failed escape. A startle was defined as a rapid, evasive movement, generally short in distance, away from the predator directed towards escape or avoidance. An approach was any distinct, investigatory movement towards the predator. A failed escape was when the predator caught and killed the fingerling, while an escape was when the predator captured but mishandled the fingerling resulting in its escape. Predator behaviours recorded were strike, approach, capture and failed capture. A strike was a distinct and fast lunge at the fingerling, approach was in this instance the predator approaching the fingerling. Capture refers to the predator capturing the prey and failed capture refers to the predator attempting but failing to

capture prey. Ultimately, the numbers of failed captures (1) and captures (4) remained too low to include in the analysis.

5.3.5 Data Analysis

Two observers recorded the data during the trial. One observer recorded the behaviour of a focal fingerling, and the behaviour of the predator was recorded simultaneously by the other observer. Data was recorded using the EthoLog program (Ottoni, 2000). Only approach data provided reliable results in the behaviours observed and thus, was the only distinct behaviour measured along with activity and zone use. To normalize data, activity was square root transformed and latency to move was log transformed. Data for area use was normally distributed and not transformed. Data was analysed using ANOVA and repeated measures ANOVA. Data were further scrutinized using a series of post-hoc tests as required (Fishers PLSD). Predator behaviour was also examined and the activity of the predators (measured as borders crossed) did not change between treatment for treatment ($F_{3, 55} = 1.29$, $P = 0.287$). All data analysis was conducted using Statview 5.0.1 (SAS Institute Inc.).

5.4 Results

In a behavioural interaction trial with a predator at liberty in semi-natural conditions with conditioned *M. novemaculeata* fingerlings, the latency to move after a predator had been introduced to the arena was investigated. A significant effect of treatment on latency to move was found ($F_{3, 55} = 3.46$, $P = 0.022$) (Fig. 5.2). Post hoc tests showed fish from the live predator treatment were significantly faster to move than those from the predator scent (Fisher's PLSD, $F_{3, 55} = 3.46$, $P = 0.003$) and the control (Fisher's PLSD, $F_{3, 55} = 3.46$, $P = 0.026$) treatments.

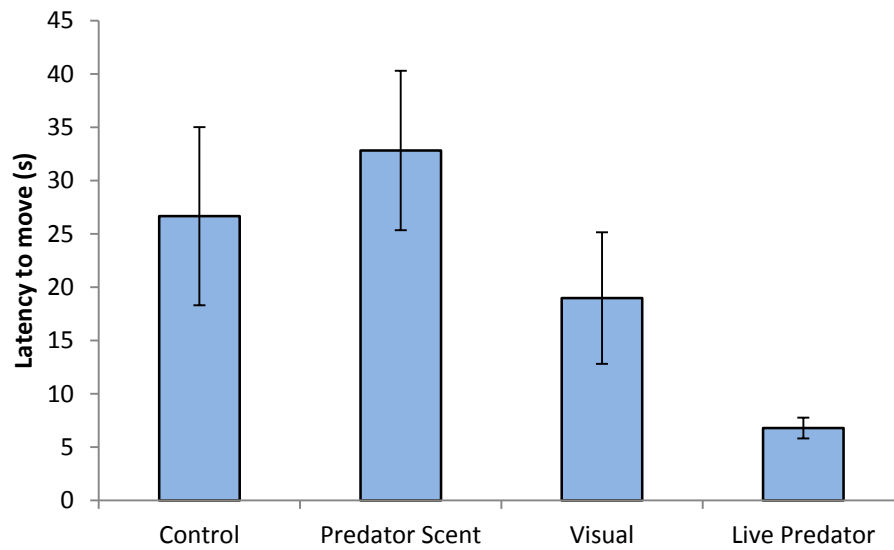


FIG. 5.2. Mean (\pm SE) Latency of conditioned fingerlings to move, in seconds, in a behavioural interaction trial with a predator at liberty in semi-natural conditions. Fingerlings were conditioned with one of four treatment; live predator, predator scent, visual cues and control.

Zone use was also investigated using rmANOVA for data in 5 minute observations, up to a total of 20 minutes. The average time spent in the planted zone by fingerlings during this 20 minutes was found to have no main treatment effect ($F_{3, 165} = 0.38$, $P = 0.765$), time effect ($F_{3, 165} = 2.43$, $P = 0.067$) or treatment by time interaction ($F_{9, 165} = 0.76$, $P = 0.655$) (Fig. 5.3).

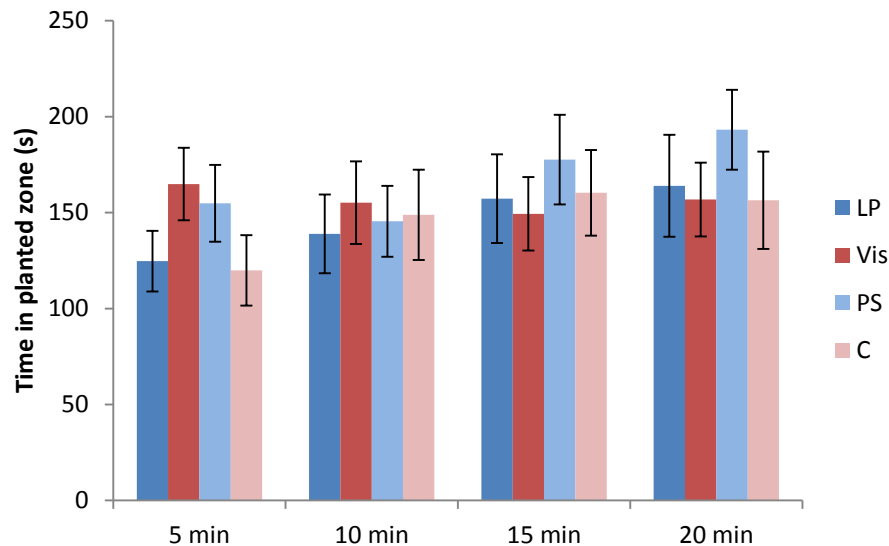


FIG. 5.3. Mean time spent in planted zone (\pm SE) during the survivorship test by conditioned Australian bass fingerlings. Fingerlings were conditioned with one of four treatments; live predator (LP), visual cues (Vis), predator scent (PS) and control (C).

No main effect of treatment was evident for border crossings (activity) by conditioned fingerlings ($F_{3, 165} = 1.78$, $P = 0.163$) (Fig. 5.4). A strong overall effect of time on the number of border crossings by conditioned fish was found ($F_{3, 165} = 4.66$, $P = 0.004$) with fish tending to recover and reduce activity over time. However, no treatment by time interaction was found for activity levels ($F_{9, 165} = 1.22$, $P = 0.283$). Pairwise post hoc comparisons showed that the fish in the predator scent treatment were less active than those in the visual only treatment ($F_{3, 81} = 3.55$, $P = 0.018$). To further investigate the data, a one way ANOVA was performed for activity levels during the first 5 min interval. While no effect of treatment was found, post hoc tests indicate the live predator treatment was significantly more active than the control treatment (Fisher's PLSD, $F_{3, 55} = 1.77$, $P = 0.038$).

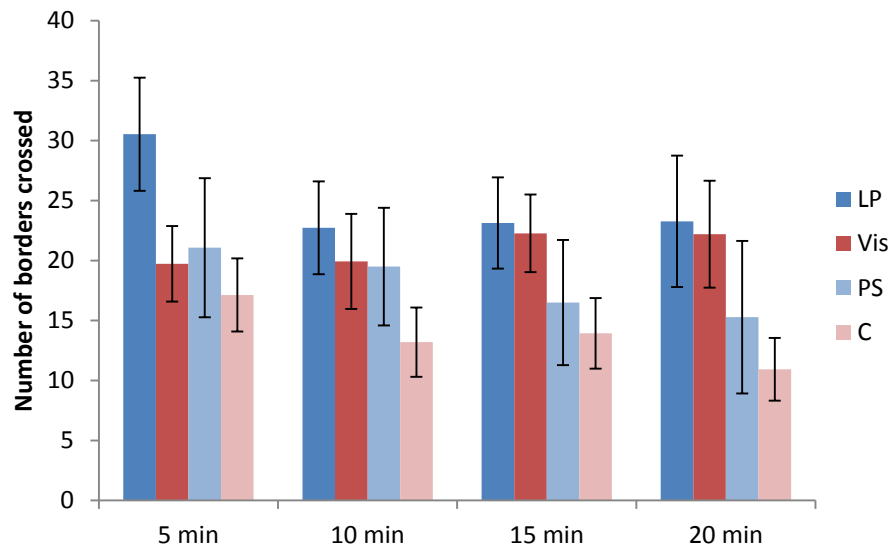


FIG. 5.4 Mean number of borders crossed (\pm SE) by conditioned fingerlings. Fingerlings were conditioned with one of four treatments; live predator (LP), visual cues (Vis), predator scent (PS) and control (C).

5.5 Discussion

Conditioning the fingerlings enhanced predator recognition and anti-predator responses, and using different cues during the conditioning process resulted in a graded range of responses. The use of multiple cues during conditioning (live predator treatment) appeared to elicit the greatest response from the *M. novemaculeata* fingerlings. Furthermore, movement rather than habitat use appeared to be the more sensitive behavioural assay as illustrated by the large difference between the treatments. Whilst habitat use varied with only modest differences in patterns of behaviour between treatments, latency of the fingerlings to move and their activity levels showed results that were generally consistent in pattern.

Conditioning the fingerlings using different predator cues created a graded effect in anti-predator response. Fingerlings from the live predator treatment showed the greatest range of response to the

predator across a range of variables. They showed high levels of activity and were the fastest to move, followed by fingerlings conditioned using visual cues. The fingerlings from the control and Predator Scent treatment were largely equal in latency to move. All treatments showed recovery over time, however, the fingerlings from the live predator and visual cues treatments always remained more active. Whilst a combined use of visual and olfactory cues (live predator treatment) elicited the most intense response, using visual cues only also appears to have altered their behaviour. These results suggest that experience with the visual cues of a predator during conditioning elicits the strongest response to a predator in later exposures.

In this experiment the fish were in close proximity with a predator which may account for the strong response to visual cues. Furthermore, the fingerlings conditioned with visual cues may have been more experienced with this sensory modality, which could also potentially alter their behaviour. Clearly vision is important in detecting predators in this species, but it is enhanced with the addition of other cues. A number of studies have also noted an additive effect in response when both visual and chemical cues are used together. For instance, star gobies *Asterropteryx semipunctatus* (Rüppell 1830) exposed to visual or chemical cues alone reacted to a similar degree, yet presented together gobies showed a greater reaction (McCormick and Manassa, 2008). Likewise, Juvenile Atlantic salmon *Salmo salar* (L. 1758) use both visual and chemical information in a combined way when exposed to a novel stimulus (Kim *et al.*, 2009). The addition of visual cues to chemical cues also caused a change in response in Anemonefish *Amphiprion percula* (Lacepède 1802), suggesting a use of combined information (Manassa *et al.*, 2013). This is likely due to the reinforcing effect of different cues. Whilst chemical cues may serve as a general avoidance warning, visual cues provide specific details, and provided together they form a more complete picture about potential threat posed by the predator. An example of this is seen in slimy sculpins *Cottus cognatus* (Richardson 1836) where avoidance of a threat was displayed when only chemical cues were available, yet when visual information was also available, avoidance was only exercised if the predator was large enough to

pose a threat (Chivers *et al.*, 2001). Thus, the addition of visual information on top of olfactory information gives more detailed account and may clarify the level of associated risk.

From the range of predator cues available to the fingerlings the type and intensity of the response may result from them adhering to a threat sensitive pattern. The threat sensitive hypothesis predicts that the intensity of the response will match the degree of threat (Helfman, 1989). If different cues are indicative of, or inform of different levels of threat it follows then that conditioning with different cues will result in differences in response. A predator that is close enough to be visually observed is likely to represent a greater threat than a predator that is not in such close proximity. This may account for the more intense response from fingerlings conditioned with a visual component, as the visual presence of a predator may represent a greater level of threat. Ferrari *et al.* (2005) noted that fathead minnows *Pimephales promelas* (Rafinesque 1820) show a more intense reaction to high risk predation cues and that this intensity of response is retained after a conditioning phase in later trials. The fingerlings from this study may be responding similarly, with a response that matches the level of threat perceived during conditioning.

Potentially, the differences between treatments may also simply reflect a species preference for the use of visual cues. The difference in patterns of activity, when compared with refuge use, also suggests this. During the trials the fingerlings from all treatments frequently approached and inspected the predator (pers. observation) rather than seeking refuge. This is consistent with the trend for visual cues being highly valuable when assessing risk. Smith and Belk (2001) suggested mosquitofish *G. affinis* rely upon visual information more during dangerous activities, such as predator inspection. Similarly Brown & Warburton (1999) found that rainbowfish *Melanotaenia eachamensis* (Allen & Cross 1982) frequently approached a predator on the far side of a mesh partition. It is possible that *M. novemaculeata* preferentially use visual cues over olfactory cues. Indeed, the morphological characteristics and diet of the species are considered to be consistent with those of euryphagic carnivores who feed by sight (Harris, 1985). This sensory preference may

result from the habitat biases of the species. If *M. novemaculeata* are often found in habitats where visual cues are generally not obstructed, visual cues are likely to be a reliable source of information. However, *M. novemaculeata* inhabit a wide variety of habitats (Harris, 1985; 1988) and cover from aquatic vegetation is favoured by this species (Allen *et al.*, 2002), so use of visual cues in their habitats may be more complicated or context specific than a fixed preference for one sensory modality over another.

The difference observed between treatments suggests that predator conditioning does facilitate learned predator recognition in this species and that it could be employed in behavioural remediation programs. In a life skills training program, investigating the relative roles of each cue will assist in enhancing and fine-tuning the approach. Interest in pre-release training has been rising for many years and an increasing body of evidence has demonstrated it is an effective way of improving anti-predator behaviour and survival of stocked fish (Brown and Laland, 2001). Furthermore, efforts have been made to show this can be achieved on a large scale, industrial scale (Olson *et al.*, 2012). However, this practice is relatively unstudied in Australian species. A notable exception has been the research performed by scientists for the MDBA, who examined predator conditioning in a range of species of Australian native freshwater fish (Hutchison *et al.*, 2012). They found predator conditioning could achieve a change in behaviour in all cases, although the details varied between species. Furthermore, they showed that survival of *M. peelii* after release was substantially improved in conditioned fingerlings, demonstrating the application of such procedures does equate to improvements in stocking efforts. Each year large numbers of *M. novemaculeata* are produced for stocking into dams and impoundments. Given the investment and popularity of the species, it would be logical to investigate any means of improving stocking success. The results shown herein highlight the plastic nature of learned predator recognition and indicate the potential for pre-release training to be implemented in this species, particularly if they are to be released into areas where they might experience heavy predation by larger fish.

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Chapter 6

Predator conditioning in hatchery reared Trout cod *Machullochella* *maquariensis*: The roles of visual and olfactory cues in learned predator recognition

This chapter is formatted for publication in the Journal of Fish Biology. All figures and tables are embedded in text.

6.1 Abstract

The ability to recognise and respond to the threat of a predator is an essential survival skill in prey animals. Fish reared in hatcheries are known to exhibit a number of behavioural deficits owing to the unnatural rearing environment, and one of their primary shortfalls is a lack of anti-predator skills. Fisheries scientists have proposed the practice of conditioning fish to react aversively to predator cues prior to release as a potential remedial technique. This is generally done by creating associations between damage-released conspecific alarm cues and predator cues. Here we investigate the relative roles of visual and olfactory predator cue on learned predator recognition in juvenile, hatchery reared trout cod *Machullochella maquariensis* in a series of laboratory trials. Results indicate that conditioning the fingerlings using predator cues elicited a change in behaviour that varied with the different modalities. Conditioning using a live predator facilitated the most intense behavioural changes, with a graded response seen when only visual or olfactory cues were used, suggesting a threat sensitive approach to learning and predator recognition. Furthermore, conditioning the fingerlings resulted in an adjustment to background behaviour, where increased vigilance was shown both before and after the addition of a predator cue. The results demonstrate there is the potential for life skills training to be utilised in this species prior to release, which may potentially improve their survival post-release.

Keywords: trout cod, *Machullochella maquariensis*, hatchery rearing, chemical cues, learned predator recognition, threat sensitive.

6.2 Introduction

The ability to recognise and react appropriately to the threat of predation is vital to survival of prey animals. It is well established that predator recognition has a significant learned component (Brown and Chivers, 2007; Kelley and Magurran, 2007). By having a large learned component to predator recognition, the anti-predator responses of an individual can be tailored to unique sets of environmental conditions. These behaviours are often acquired through individual experience with contemporary predators and related cues, which may occur in both visual and olfactory form. This is generally through the association of damaged conspecifics and a predator cue (Brown and Chivers, 2007). From these cues the prey may make an assessment of risk and moderate their behaviour accordingly (Lima and Dill, 1990).

The threat sensitive hypothesis predicts that prey will match the intensity of their response to the perceived level of threat (Helfman, 1989). The different cues available to fish when making assessments of risk differ in the properties and benefits unique to them. Visual cues provide an array of information about the predator that are generally detailed and temporally specific such as size (Chivers *et al.*, 2001) or hunger (Licht, 1989). For example, when presented with a series of models of increasing realism, rainbowfish *Melanotaenia eachamensis* (Allen & Cross 1982) reacted most to a realistic model that moved (Brown and Warburton, 1997). On the other hand, while olfactory cues are temporally less reliable, they can provide information at a greater and thus safer distance (Brown and Chivers, 2007; Ferrari *et al.*, 2010b). The array of cues available to fish can be used in a threat sensitive way, and elicit a variety of responses. Rather than responding in a fixed way to the different modalities, the prey may exhibit anti-predator responses that are graded or additive, where the response matches the perceived level of risk (Ward and Mehner, 2010). Coral reef fish vary in their utilization of visual and chemical cues with a greater relative importance placed on visual cues (Marsh-Hunkin *et al.*, 2013). Conversely, McCormick and Manassa (2008) found coral reef

gobies *Asterropteryx semipunctatus* (Rüppell 1830) to respond more intensely when both visual and chemical cues were presented.

Experience with predators is another factor likely to contribute to an individual's response to predators. Predator naïve fish are able to improve their anti-predator response rapidly with experience (Berejikian, 1995; Brown and Warburton, 1999). However, rearing fish in hatcheries denies them the opportunity to learn about predators firsthand. Pre-release training has made use of the learning abilities of fish in attempts to facilitate learned predator recognition in fish destined for release in stocking programs (Brown and Laland, 2001). While such training regimes are commonly employed in terrestrial conservation programs, they are rarely applied to fish (Brown and Day, 2002). Nevertheless, there is a host of evidence that learning plays a key role in the development of fish behaviour (Brown *et al.*, 2011). Associative learning can be effectively used to condition an evasive response to predator cues. This method has proven successful in enhancing predator recognition in predator naïve fish in laboratory studies (Brown and Smith, 1998) and in the field (Lönnerstedt *et al.*, 2012).

Restocking programs are often used for two primary purposes: 1) conservation management and 2) fisheries enhancement. Both rely on fish surviving the restocking process and growing to a size where they either reproduce or recruit into the fishery. Either scenario requires the fish to live for a considerable length of time in the wild. However, data from around the world has shown that fewer than 5% of hatchery-reared fish survive (Brown and Day, 2002). Thus any program that can enhance the post-release survival of hatchery-reared fish by just incremental amounts will make a substantial difference to the efficacy of restocking. Despite Australia having extensive stocking programs, there is limited data on the survival of stocked fish and only a single investigation has been conducted on pre-release training in Australian fish. Research funded by the Murray-Darling Basin Authority (Hutchison *et al.*, 2012) showed predator conditioning of hatchery reared Murray cod *Maccullochella peelii* (Mitchell 1838), silver perch *Bidyanus bidyanus* (Mitchell 1838) and catfish *Tandanus tandanus*

(Mitchell 1838) using conspecific skin extract was able to produce a change in behaviour in response to a predator during laboratory tests in all three species. Murray cod *M. peelii* stocking trials showed pre-release training was also able to improve the post-release survival.

Trout cod *Machullochella macquariensis* (Cuvier 1829) is an Australian native freshwater fish that was formerly widespread in the southern Murray- Darling system. They are a large, predatory species that inhabits flowing waters with woody debris (Lintermans, 2007). The streams featuring characteristics preferred by *M. macquariensis* are limited in number (Nicol *et al.*, 2007) and such habitat preferences are likely to restrict distribution of the species. Historically, the distribution of this species has extended from the southern tributaries of the Murray to the Macquarie River (DPI, 2006). However, for many years only three self-sustaining populations remained; Cataract Dam, Sevens Creek and the Murray River between Yarrawonga and Barmah (Lintermans, 2007). The Cataract Dam and Sevens Creek populations being stocked, translocated populations. With ongoing stocking efforts, a number of other sites are closer to being considered 'self-sustaining' populations (Koehn *et al.*, 2013).

A number of *M. macquariensis* fingerlings have been produced in government hatcheries in recent years for stocking programs. They are easily produced and reared in hatcheries, however, licencing is restricted to government facilities to ensure ongoing quality and maintained quality of broodstock. The number of stocked *M. macquariensis* since 1987 has totalled over 1.5 million (Koehn *et al.*, 2013). While there have been some instances of success in stocked trout cod, and indeed, some populations depend upon replenishment by stocking, widespread success has been modest. In a study by Ebner *et al* (2007), two year old hatchery-reared *M. macquariensis* released into the Murrumbidgee and Cotter Rivers experienced rapid mortality and almost complete mortality after a period of 7 months (1 surviving individual). Nonetheless, the stocking of *M. macquariensis* has been considered a relative success compared with other reintroduction programs (Lyon *et al.*, 2012) even though data is mostly lacking.

Whilst there are likely to be several factors that determine the survival of released fingerlings, behavioural deficits have been recognised as a primary factor in the failure of stocking attempts (Olla *et al.*, 1998). The behavioural deficits are largely due to the unnatural rearing environment in which the fish are cultured, and one of the major elements absent in the hatchery environment is pressure from predators (Olla *et al.*, 1998; Brown, 2006). Hatchery fish are subsequently behaviourally ill-equipped to deal with the threat of predation. Here, the use of chemical and visual cues to condition predator recognition and anti-predator response were examined in a laboratory setting using hatchery reared trout cod *M. macquariensis*. Two main questions were addressed in this study 1) does conditioning with damage-released conspecific cues paired with predator cues result in acquired recognition of a novel predator and 2) what cues, or combination of cues, best enhances the anti-predator behaviour of hatchery reared, juvenile trout cod. The outcomes of this research not only add to our limited knowledge of the anti-predator responses of this species, but also highlight possible avenues for life-skills training for hatchery-reared individuals.

6.3 Methods

6.3.1 Subjects and Housing

Subjects were young of the year trout cod *M. macquariensis* fingerlings of around 5.5cm TL. They were produced by NSW Industry and Investments, Narrandera where they had been reared in outdoor dams. The fingerlings that were used were the first generation offspring of wild caught broodstock. The fingerlings were transported to Macquarie University, Sydney, overnight in oxygenated water packed in Styrofoam boxes. Upon arrival at Macquarie University they were transferred into outdoor ponds (capacity 1530l) at a density of 100 fish per pond. Ponds were kept at ambient temperature (average 22°C) and filled with aged, de-chlorinated water. The ponds were

maintained under bird netting to avoid avian predation. During the first week, the fingerlings were weaned onto flake food using bloodworms. They were fed flake food 5 days in each week and maintained in these outdoor ponds until the beginning of experiments.

The fish were moved into tanks within a laboratory for conditioning prior to testing. The conditioning tanks measured 90cm long x 35cm wide and were filled to 35cm deep. Tanks were placed flush with the wall and all other sides of the tanks (excluding the top) were covered to minimise visual interaction and prevent undue stress on the fish from the activity of lab users. The room had a 12hrs light: 12hrs dark light cycle. Each aquarium was fitted with a filter (capacity 500l/hr), gravel and heater set at 16°C. The fish were placed in these tanks the day before conditioning started, to allow them to settle.

Three wild caught, mature Spangled Perch *Leiopotherapon unicolor* (Günther 1859) with an approximate length of 15cm were used as predators. Spangled perch *L. unicolor* have a wide-spread distribution in the Murray-Darling Basin and are renowned for their vigorous predatory nature. Fish were kept individually in 60L tanks, with gravel substrate and a filter of 500l/hr capacity. The tanks were given a 1/3 volume water change each fortnight. The *L. unicolor* were fed on prawn 5 days in every week prior to the experiment. They were fed only leading up to the conditioning phase, and not fed during the conditioning. After the conditioning, they were placed back in their housing tank and their feeding resumed.

6.3.2 Conspecific Extract (CE)

Conspecific Extract (CE) was generated by homogenising whole conspecifics and passing the liquid through a filter (see Chapter 2, Methods 2.3.3.1 for details).

6.3.3 Conditioning treatments

A total of 16 replicate fish were conditioned per treatment. The fingerlings were conditioned to predator cues in groups of 8 fingerlings per batch. Two batches of eight were conditioned per treatment to achieve the total of 16 fish per treatment. Fingerlings were conditioned in four separate tanks. Each tank was designated as one of 4 treatments (see below). Fish were exposed 3 times per day to the predator stimuli. This was done each day between the hours of 9am – 10am, noon – 1pm and 3pm – 4pm for 3 days (a total of 9 exposures). Extensive pilot studies suggested that this was the most effective training regime. Immediately after the predator conditioning, the fingerlings underwent 10 days of foraging conditioning, to accustom them to a routine of feeding prior to testing. The four treatments were as follows;

6.3.3.1 Predator scent combined with CE

The predator scent combined with CE treatments (henceforth abbreviated to predator scent treatment) received a combination of conspecific extract and predator scent only, with visual cues excluded. Water that contained the chemical signatures of the predator was used as predator scent. Immediately prior to exposure, 20ml of water was collected from a 60l tank containing a single *L. unicolor* that had been housed in the tank. This volume was similar to that used in studies by Brown & Smith (1998). The tank had no water changes or cleaning done for a week prior to the conditioning stage. The scent was added to the conditioning tank immediately followed by 20ml CE. This is a typical method of entraining predator recognition in a wide range of species (Brown *et al.*, 2011). At the completion of each conditioning the tank was drained completely, cleaned and refilled with de-chlorinated tap water, ready for the next group to be conditioned.

6.3.3.2 Live predator combined with CE

During this treatment (henceforth abbreviated to live predator treatment) a live predator was placed behind a barrier within the conditioning tank. A partition made from fine mesh (2mm²) was placed at 1/3 of the length of the tank along, creating two sections; a large section (measurements 62cm x 35cm depth x 35cm wide) and a small section (measurements 31cm x 35cm depth x 35cm wide). The predator was placed in the small section of the tank whilst the fingerlings were placed in the large section. Water was able to pass through the partition, allowing circulation of water and chemical cues. An opaque partition placed adjacent to the mesh was attached to a pulley, and could be lifted remotely. This was lifted at each exposure, for a duration of 15min, to allow visual contact with the predator. This was done in conjunction with the addition of 20ml CE. The predator was removed over night to prevent it attempting to escape. At the completion of each conditioning the tank was drained completely, cleaned and refilled with de-chlorinated tap water.

6.3.3.3 Visual cues only combined with CE

This treatment (henceforth abbreviated to visual cue treatment) received a combination of CE and visual contact with the predator, in the absence of any chemical cues from the predator. The fingerlings were exposed to visual cues from the predator in conjunction with CE, in the absence of predator scent. This was done by keeping the fingerlings in a tank covered with black plastic on three sides to limit any other visual cues. On one short side, a tank of the same dimensions containing a predator was placed flush with the fingerling tank. An opaque Perspex barrier was placed between the two tanks and was attached to a remote pulley that allowed it to be lifted, providing visual contact between the two tanks. At the designated conditioning times, the predator was gently guided to the end of its tank closest to the fingerlings and contained there by placement of a clear barrier 30cm away from the end of the tank. The visual barrier between the two tanks was lifted and the CE carefully added to the fingerlings tank at the end nearest the predator. The barrier remained

up for 15min. At the end of the 15min, it was lowered and the predator was released by removing the clear partition. At the completion of each conditioning the fingerlings tank was drained completely, cleaned and refilled with de-chlorinated tap water.

6.3.3.4 Control

This treatment was representative of standard hatchery fish used for stocking and therefore received no conditioning. The fingerlings were placed in conditioning tanks of the same dimensions and water volume as the other treatments the day prior to when conditioning would begin. In place of the exposures, the control group were simply left in the aquaria for the equivalent amount of time. Before the next group of fish was conditioned, the tank was drained completely, cleaned and refilled with de-chlorinated tap water.

6.3.4 Foraging test conditioning

On the morning after the predator conditioning had been completed, the fingerlings were individually placed in the foraging tanks. Each foraging tank measured 46cm x 24cm and was filled to a water level of 20cm. Each tank was covered around the sides with black plastic to prevent visual disturbance and was aerated using a small air stone. The foraging tanks were split into thirds by a mark on the base, and were furnished with a hide (a small, square black tube; 5cm x 5cm x 12cm) placed in the centre. The benthos was covered with river gravel. At one end of the tank was placed a tube where water or scent could be added to the tank (Fig. 6. 1). The fish were kept in these tanks for 10 days. Each day, at 9am and again at 3pm, the fish were fed approximately 8 bloodworms delivered by eyedropper directly next to the tubing into the tank. After 20min any uneaten bloodworms were removed from the tank. All fish fed during this training regime. After the 10 days

the fish had had 20 foraging exposures and were accustomed to foraging on bloodworms at that end of the tank.

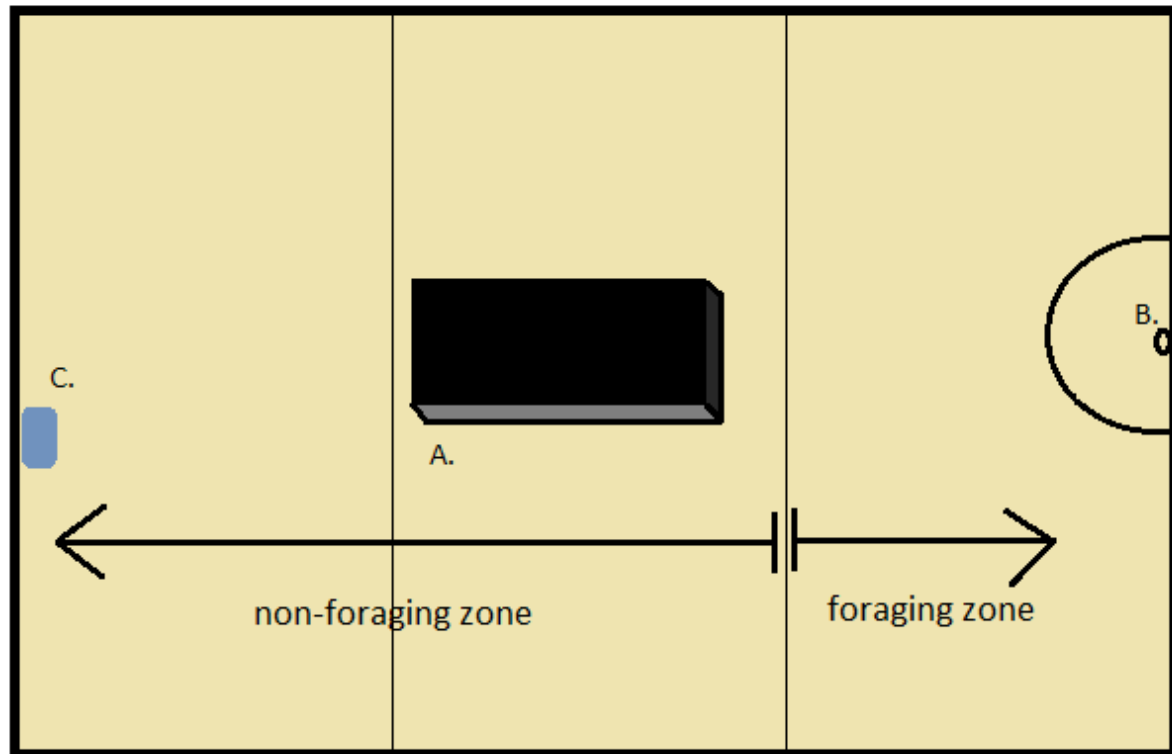


FIG. 6.1 The foraging tank measured 46cm x 24cm and was filled to a depth of 20cm. A) A hide (a small, square black tube; 5cm x 5cm x 12cm) placed in the central third of the tank. B) Tube for adding predator cue. Bloodworms were also dispensed in this location. C) Airstone for water circulation.

6.3.5 Foraging test; experimental procedure

Following the foraging conditioning phase, the fish had 1 day with no feeding to promote foraging behaviour during testing. Foraging testing was done over 2 days, with the fish receiving 1 test per day. The foraging test had 2 stimuli, Predator scented and unscented water, which were tested separately, one each day. During testing, each fish was given 20ml of stimuli (either predator scented or unscented water) delivered through the tubing into the tank, followed immediately by 10 bloodworms. The behaviour of the fish was viewed remotely by camera and recorded for 20min. Use of the foraging zone and the non-foraging zone were recorded as well as use of the hide. Fish

spend most of their time hidden in the hide when not feeding and thus the latency to emerge from the hide was also measured. On the first day of testing half the fish received the predator scent and the other half received water as a control. The next day the same procedure was performed, with the test stimuli reversed.

6.3.6 Live Predator test; experimental procedure

The Predator test was performed the day following the completion of the foraging test. The experimental tank for this test measured 92cm x 51cm, filled to a depth of 31cm (Fig 6.2) and consisted of 2 compartments, separated by a clear Perspex partition placed at approximately 1/3 of the length of the tank (30cm). The smaller section was termed the predator zone. The partition had small holes drilled through it to allow some water movement, thus allowing for both chemical and visual contact of fish on either side. Gravel was placed on the bottom of the predator zone, blocking the bottom and edges of the partition to prevent fingerlings from reaching the predator zone. A small pump (capacity of 150L per hour) was also placed in the predator zone to ensure circulation of the water. The large section tank was divided into 'zones', marked on the underside of the tank with black marker. One of these zones was designated the planted zone and contained four plastic plants, evenly spaced within the zone. The other zone was designated the open zone and contained nothing. Open and planted zones were at the far end of the tank from the predator compartment to distinguish any preferences for cover as being distinct from simply maximising the distance from the predator. To control for any side preferences, the planted zone and the open zone alternated with each test. The area nearest the predator zone was termed the near zone. Entering the near zone was likely to reflect predator inspection whilst use of the planted zone was likely to indicate refuge use. In addition to the clear Perspex partition, an opaque barrier was also placed in the tank, separating the predator from the fingerlings visually. This was attached to a pulley, so it could be lifted

remotely, allowing visual contact between the fingerlings and predator. This barrier also limited the movement of chemical cues into the prey compartment.

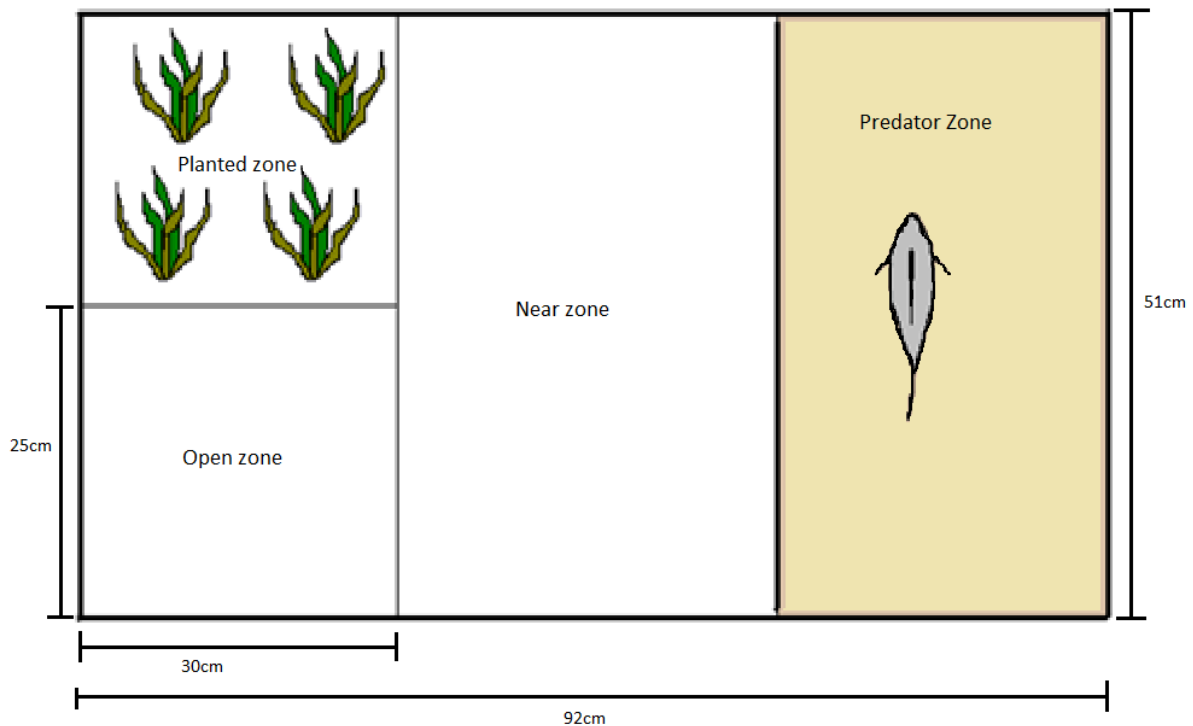


FIG. 6.2 Predator test experimental tank diagram. Measurements 92cm long x 51cm wide, filled to a depth of 31cm. The predator zone was separated from the rest of the tank by a clear, perforated partition. Gravel was placed on the base of the predator zone and the planted zone was furnished with four plastic aquarium plants.

Fingerlings were placed individually in the testing tank, in the near zone. Fish were allowed to settle for 20 min prior to recording. Their behaviour was then recorded for 20 min on an overhead camera before the opaque barrier was lifted, then for a further 20 min after the barrier was lifted, while the predator was visible. Their use of the different zones, termed area use, was recorded in real time as was the number of border crossings (a measure of activity level).

6.3.7 Data analysis

ANOVA and Repeated measures ANOVA were used to analyse the data. All data, from both the live predator test and the foraging test, was log transformed to achieve normality. The only exception to this was the data for planted area use, which was normal and thus left untransformed. The training treatments were used as independent variables and the values before and after exposure to the predator cues treated as the repeated measures. Use of covered zones by conditioned trout cod fingerlings was examined using data split into 5 min intervals, which included 5 min prior to the predator cue, and 20 min after the predator cue, for a total of 25 min. Data were further scrutinized using a series of post-hoc tests as required. Post-hoc tests used were Fisher's PLSD and one-way ANOVA. All data analysis was conducted using Statview 5.0.1 (SAS Institute Inc.).

6.4 Results

6.4.1 Foraging test

Results of a Repeated measures ANOVA indicated there was no main effect of treatment for number of emergences in conditioned fingerlings ($F_{3, 58} = 2.40$, $P = 0.077$, Fig. 6.3). There was also no significant effect of time ($F_{1, 58} = 0.31$, $P = 0.582$). A significant interaction of treatment and time, however, was present ($F_{3, 58} = 4.24$, $P = 0.009$). Fingerlings from the control treatment showed no change in behaviour between the unscented water and the predator scent. Fish conditioned in the visual cue treatment showed the only significant reduction in emergences in the presence of predator scent ($F_{3, 58} = 3.27$, $P = 0.028$). Pairwise post hoc tests (Rm ANOVA) showed that the fish in the visual cue treatment displayed a reduction in number of emergences from cover, whereas the fish in the live predator ($F_{1, 28} = 8.45$, $P = 0.007$) and predator scent ($F_{1, 28} = 7.37$, $P = 0.011$) treatments showed an increase in the number of emergences when exposed to the predator cue.

Further post-hoc one way ANOVAs were performed for both the water test and the predator scent test. These indicated the fish in the control treatment had a significantly higher number of emergences than those in the predator scent treatment in the unscented water test (Fisher's PLSD $F_{3, 58} = 2.06$, $P = 0.0196$). The control treatment fish had significantly more emergences than those conditioned in the visual cue treatment in the predator scent test (Fishers PLSD $F_{3, 58} = 3.30$, $P = 0.007$), and the fish from the live predator treatment had significantly more emergences than the fish from the visual cue treatment (Fisher's PLSD $F_{3, 58} = 3.30$, $P = 0.013$). No effect of treatment ($F_{3, 58} = 0.16$, $P = 0.926$), time ($F_{1, 58} = 0.62$, $P = 0.436$) or treatment by time interaction ($F_{3, 58} = 0.72$, $P = 0.544$) was observed on the latency of the fingerlings to first emerge from cover.

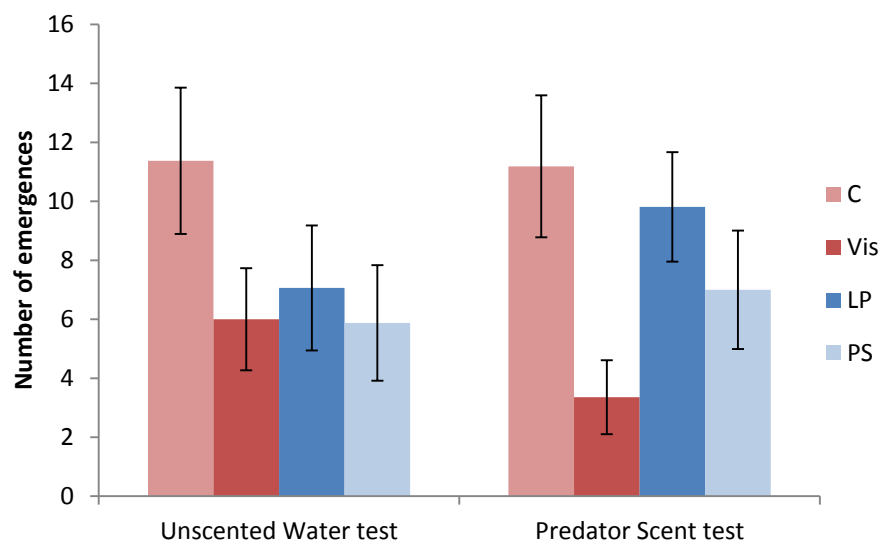


FIG. 6.3 Mean (\pm SE) number of emergences from hide of Trout cod fingerlings during the foraging test when exposure to unscented water and to water containing predator scent. Fingerlings were conditioned with one of four treatments; live predator (LP), predator scent (PS), visual cue (Vis) and control (C).

A repeated measures ANOVA was used to evaluate differences in mean duration in foraging zone and revealed no main effect of treatment ($F_{3, 58} = 2.15$, $P = 0.104$, Fig 6.4) and no effect of time ($F_{1, 58}$

= 0.60, $P = 0.441$). However, a significant interaction of treatment and time was evident ($F_{3, 58} = 3.27$, $P = 0.028$). Post hoc tests showed the fish conditioned in the control treatment reduced average duration in the foraging zone in the presence of predator scent cue ($F_{1, 15} = 6.56$, $P = 0.022$) whilst the fish from the live predator treatment increased the average duration in the foraging zone in the presence of the predator cue ($F_{1, 15} = 4.78$, $P = 0.045$). Post hoc one-way ANOVA indicated that during the unscented water test the control treatment fish showed a significantly longer average duration in the foraging zone than those in the live predator (Fisher's PLSD $F_{3, 58} = 3.21$, $P = 0.015$), predator scent (Fisher's PLSD $F_{3, 58} = 3.21$, $P = 0.012$) and visual cue treatments (Fisher's PLSD $F_{3, 58} = 3.21$, $P = 0.018$). During the predator scent test the fish from the live predator treatment showed a greater average duration in the foraging zone than the visual cue treatment fish (Fisher's PLSD $F_{3, 58} = 1.73$, $P = 0.039$).

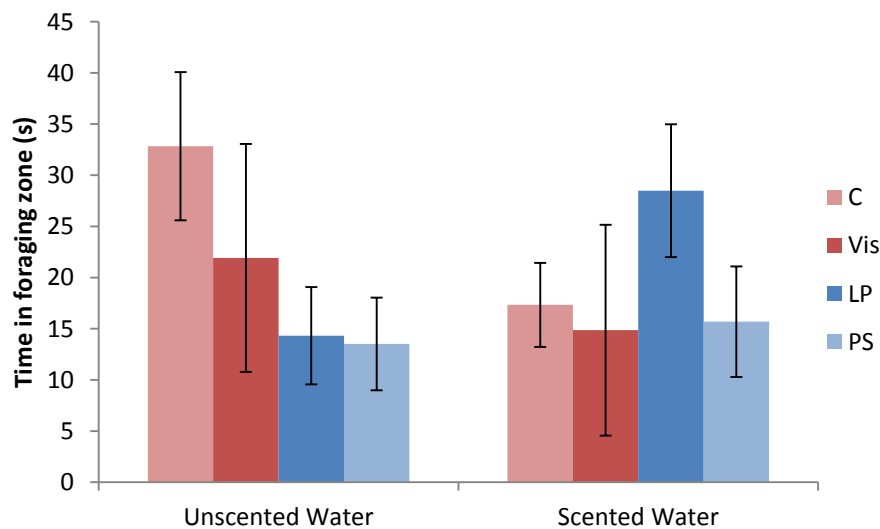


FIG. 6.4 Average duration of conditioned fingerlings in the foraging zone (\pm SE) in a test where they were presented with unscented water or with water containing predator scent. Fingerlings were conditioned with one of four treatments; live predator (LP), predator scent (PS), visual cue (Vis) and control (C).

A repeated measures ANOVA discovered no significant effect of treatment ($F_{3, 58} = 1.24, P = 0.304$), time ($F_{1, 58} = 0.96, P = 0.332$), or treatment over time ($F_{3, 58} = 1.58, P = 0.204$) on the latency of fingerlings to enter the foraging zone.

6.4.2 Live predator test

Results of the repeated measures ANOVA indicate there was no main effect of treatment for planted zone use ($F_{3, 232} = 2.48, P = 0.070$), open zone use ($F_{3, 232} = 0.83, P = 0.482$) or near zone use ($F_{3, 232} = 2.66, P = 0.057$) by conditioned fingerlings. There was, however, an effect of time for planted zone use ($F_{4, 232} = 3.23, P = 0.013$; Fig 6.5), open zone use ($F_{4, 232} = 3.21, P = 0.014$; Fig 6.6) and near zone use ($F_{4, 232} = 3.96, P = 0.004$; Fig 6.7). There was no treatment- time interaction for either the open ($F_{12, 232} = 1.32, P = 0.206$) or near ($F_{12, 232} = 1.66, P = 0.076$) zone use by conditioned fingerlings. However, an interaction between treatment and time was evident in the use of the planted zone by conditioned fingerlings ($F_{12, 232} = 1.83, P = 0.045$; Fig 6.5). Post hoc tests revealed the use of planted zone changed over time for fish in both the control ($F_{4, 60} = 2.82, P = 0.033$) and the predator scent treatments ($F_{4, 60} = 4.23, P = 0.004$), but not in the fish conditioned in the live predator and visual cues treatments ($P > 0.05$). Changes in zone use over time were also evident in the predator scent treatment fish for use of the open zone ($F_{4, 60} = 4.87, P = 0.002$) and the near zone ($F_{4, 60} = 3.94, P = 0.007$). Pair wise comparisons of treatments indicated a significant treatment –time interaction for the fingerlings in the predator scent treatment and those in the control treatment in use of the planted zone (RMANOVA: $F_{4, 120} = 3.67, P = 0.007$). The most striking difference being a greater use of cover by fish trained in the predator scent treatment prior to the predator being revealed. Predator scent treatment fish also differed from fish trained in the visual cues treatment in use of the planted zone ($F_{4, 112} = 2.56, P = 0.042$), the open zone ($F_{4, 112} = 3.26, P = 0.014$) and the near zone ($F_{4, 112} = 3.22, P = 0.015$). However, no statistically significant differences were found between the control and the other treatments.

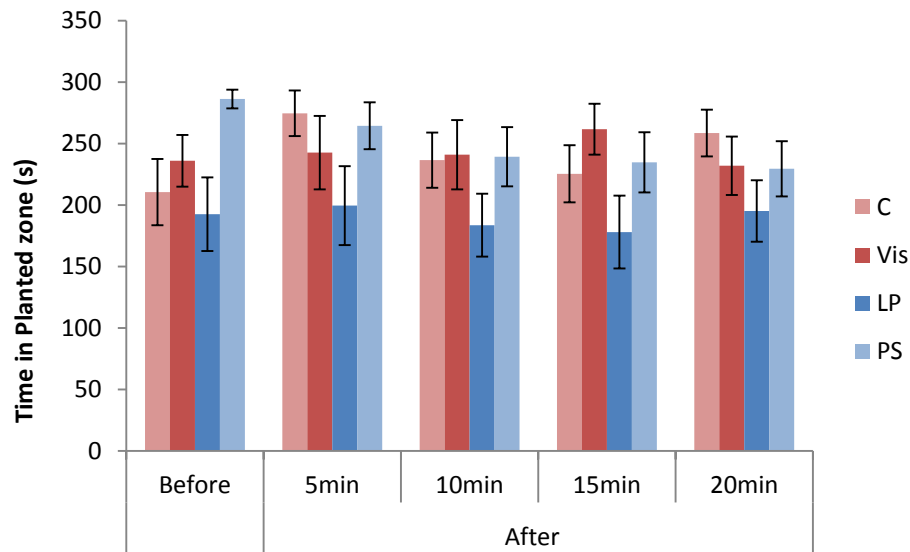


FIG. 6.5 Average (\pm SE) time spent (s) in the planted Zone, before and after the predator was revealed, by conditioned *M macquariensis* fingerlings, broken into 5 min observations. Fingerlings were conditioned with one of four treatment; live predator (LP), predator scent (PS), visual cue (Vis) and control (C).

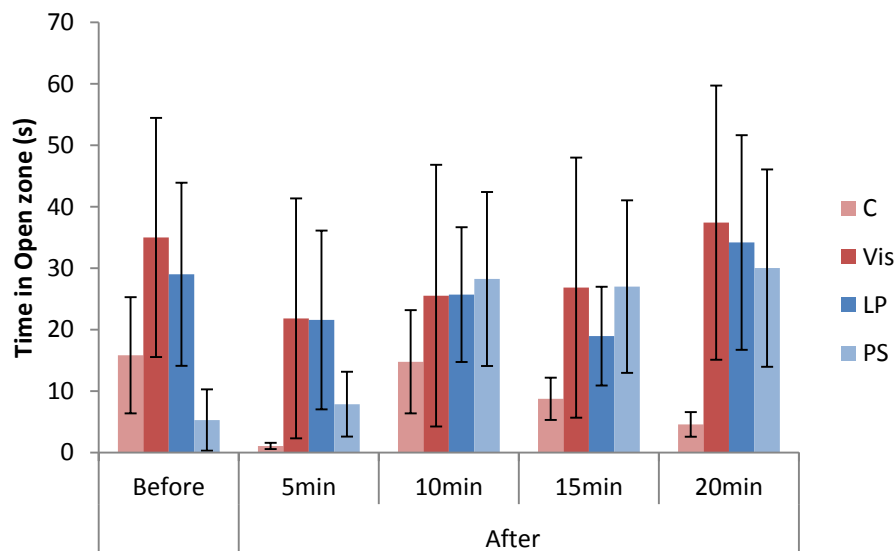


FIG. 6.6 Mean (\pm SE) time spent (s) in the open zone, before and after the predator was revealed, by conditioned *M macquariensis* fingerlings, broken into 5 min observations. Fingerlings were conditioned with one of four treatment; live predator (LP), predator scent (PS), visual cue (Vis) and control (C).

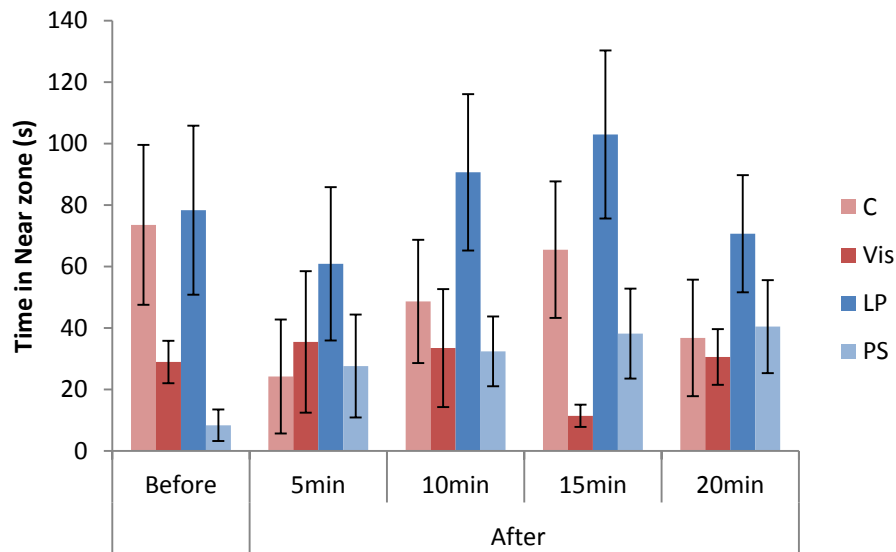


FIG. 6.7 Mean (\pm SE) time spent (s) in the near zone, before and after the predator was revealed, by conditioned *M. macquariensis* fingerlings, broken into 5 min observations. Fingerlings were conditioned with one of four treatments; live predator (LP), predator scent (PS), visual cue (Vis) and control (C).

No main effect of treatment was found in activity levels of conditioned fingerlings ($F_{3, 232} = 0.66$, $P = 0.582$, Fig. 6.8), but a strong effect of time was evident ($F_{4, 232} = 8.44$, $P < 0.0001$). A significant interaction of treatment and time, was also evident following exposure to the predator ($F_{12, 232} = 2.10$, $P = 0.018$). Post hoc tests indicate that fish from the control, predator scent and visual cue treatments increased activity significantly after exposure to the predator ($F_{4,60} = 2.61$, $P = 0.044$, $F_{4,60} = 7.38$, $P < 0.0001$; $F_{4, 52} = 2.79$, $P = 0.036$ respectively) while fish trained using the live predator treatment did not. Further pairwise comparisons showed the fingerlings from the predator scent treatment showed less activity, particularly prior to the predator being revealed, than the visual cue ($F_{4,112} = 5.10$, $P = 0.001$), and live predator treatment fingerlings ($F_{4,120} = 2.64$, $P = 0.037$).

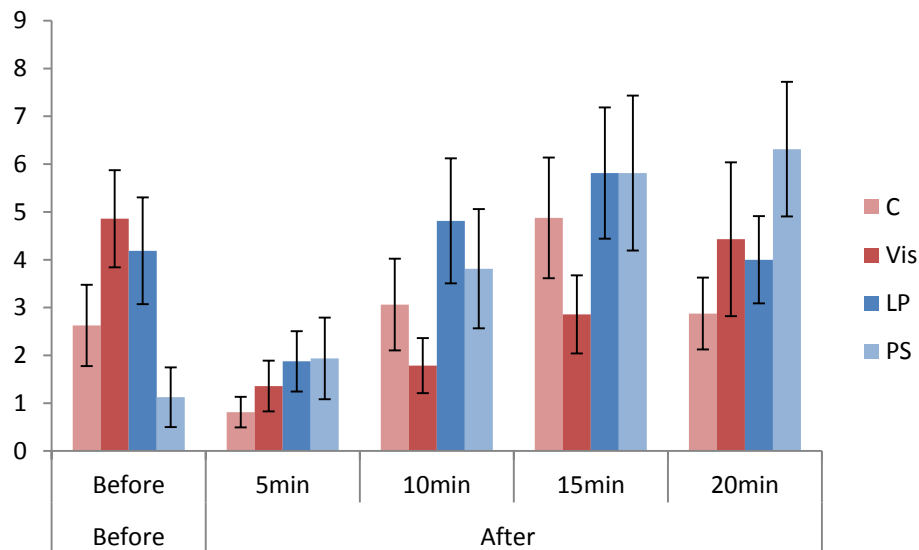


FIG. 6.8. Mean (\pm SE) number of border crossings (activity) of conditioned *M. macquariensis* fingerlings before and after exposure to a predator. Fingerlings were conditioned with one of four treatment; live predator (LP), predator scent (PS), visual cue (Vis) and control (C).

6.5 Discussion

Conditioned *M. macquariensis* fingerlings were able to learn to recognise a *L. unicolor* predator and adjusted their anti-predator behaviour in its presence. A graduated range of responses from the conditioned fingerlings was observed depending on the conditioning treatment and test used. This range of responses included changes in area use, predator inspection and activity levels. It is possible this demonstrates a flexible approach to anti-predator behaviour, as anticipated by the threat sensitive hypothesis which predicts that prey should match their anti-predator behaviour to the perceived level of predation risk (Helfman, 1989). This may vary as a function of both prior history with predators and the cues that are presently detected (for instance visual vs. olfactory). Predator threats come in a variety of forms and intensities, and fish have the capacity to learn a vast repertoire of defences and modifications to their behaviour that allow them to adjust accordingly

(Kelley and Magurran, 2007). Given this, it is not surprising that differences in predator cues during learning phases resulted in a range of responses.

6.5.1 Foraging test

During the foraging tests, the fingerlings conditioned using a live predator were more likely to emerge from a hide in the presence of predator scent than they did with unscented water. This increase in emergence is likely another example of predator inspection behaviour, seen exclusively in fingerlings trained using a live predator.

The fingerlings from the predator scent treatment showed little change in their propensity to emerge from cover between unscented water and the predator scent. They did, however, have a lower propensity to emerge than the control treatment, as did the fingerlings from the visual cue treatment. Once again, the conditioned fingerlings behaved differently from the control fingerlings, suggesting that conditioning can alter their behaviour and vigilance whether predator cues are present or not. In natural environments, enhanced vigilance may increase survival in terms of reduced predation, but it likely comes at a cost of lost foraging opportunities.

The fingerlings from the control treatment, in contrast, showed a large decrease in use of the foraging zone in the presence of predator scent suggesting an underlying innate recognition of predator scent. Whilst the fingerlings from the predator scent and visual cue treatments were fairly static in foraging zone use, both in the presence of predator cue and not, they both spent less time in the foraging zone than the fingerlings from the control treatment in unscented water. Therefore, whilst it appears there is a degree of innate recognition of predator scent shown by the control fingerlings, this is clearly modified by varying the individuals experience during conditioning. Foraging behaviour was adjusted, not in response to a present cue, but due to prior history with predator cues. Foam *et al.* (2005a) found convict cichlids *Amatitlania nigrofasciata* (Günther 1867) exposed to sub threshold levels of predator risk did not show an overt behavioural response but did

change their foraging behaviour accordingly. It is possible the fingerlings from the visual cue and predator scent treatments have altered their background behaviour as a consequence of the threat levels experienced during conditioning.

The fingerlings from the live predator treatment increased use of the foraging zone significantly in the presence of predator scent. Potentially, these fingerlings, after training with multiple predator cues, perceived a lower level of risk when presented with predator scent alone. It is well documented that predator behaviour is a good indicator of predation threat, for example, guppies *Poecilia reticulata* (Peters 1859) can tell the difference between a hungry and satiated predator (Licht, 1989), and this is information that is best acquired visually. Likewise, there is evidence that predator recognition from multiple cues is more effective than from singular cues. Lehtiniemi (2005) found pike *Esox lucius* (L. 1758) and stickleback *Gasterosteus aculeatus* (L. 1758) larvae can detect a predator using olfactory cues alone but a greater and more varied response was gained using a combination of visual and olfactory cues. Whilst olfactory cues on their own may function as a warning, visual and olfactory cues together provide more detailed information, reinforcing one another. Behavioural responses to predator cues are likely to be context specific and dependent upon the local environment. For instance, in a study by Brown and Warburton (1997), rainbowfish *M. eachamensis* from structured habitats tended to respond to a predator by seeking refuge in vegetation, whereas *M. eachamensis* from open areas increased schooling. Likewise, turbidity changes the visual information received by fish (Ferrari *et al.*, 2010a). In the absence of visual information, fish may rely more heavily on olfactory cues (Hartman and Abrahams, 2000).

It appears that conditioning fingerlings with multiple cues (ie using a live predator) elicits the most dynamic range of responses. Smith and Belk (2001) found multiple cues improved anti-predator response, but this was dependant on the cues and behaviours scrutinised. The dynamic nature of anti-predator responses shown here suggests a graduated response that is relative to perceived threat. Ferrari *et al.* (2005) found minnows *Pimephales promelas* (Rafinesque 1820) learned

predator response in a manner that reflected threat levels during training, a stronger response was associated with high risk predation cues. From the present study it is hard to say whether the change in behaviour resulted more from experience with a diversity of cues or from a response that matched background threat levels (ie conditioning). However, we can infer that that both types of predator cue, both visual and olfactory, play a role in learned predator recognition in this species.

6.5.2 Live predator test

When presented with a live predator, the fingerlings conditioned using a live predator were the only treatment to show a significant increase in near zone use. This change in behaviour could best be described as predator inspection. The fingerlings from the live predator treatment also showed less use of cover, both before and after the predator was revealed. They were also the only treatment that did not significantly change their activity levels over time. Predator inspection behaviour has been shown to provide valuable information about predation threat (Murphy and Pitcher, 1997; Brown and Godin, 1999) and can assist in deterring predators (Godin and Davis, 1995), however, it does come with inherent risks. Dugatkin (1992) found predator inspection in *P. reticulata* was a predictor of mortality, where high levels of inspection behaviour correlated with higher mortality. Smith and Belk (2001) noted that risky behaviours, such as inspection, rely more heavily on information from visual cues. The fingerlings from the live predator treatment were experienced with both visual and olfactory cues from the predator and are likely to make use of both these cues to determine threat levels and were able to judge present risk and partake in predator inspection.

The fingerlings from the visual cue treatment showed an intermediate response with no great change in pattern of cover use. In contrast to the fingerlings from the live predator treatment, the fingerlings from the predator scent treatment showed the greatest use of cover, both before and after the presentation of a predator before beginning to recover, suggesting that long term changes in their behaviour were induced during the training regime. Association with woody debris is common in many Australian native fish. The habitats preferred by *M. macquariensis* are often highly

structured (Nicol *et al.*, 2007) and *M. macquariensis* are usually found near a snag or woody habitat (Gowns *et al.*, 2004). The physical characteristics of the habitat are likely to impact on the methods of predator detection used. In such a situation where vision is limited, the fish may rely more heavily on the use of olfactory cues in gathering information about potential threats and in the presence of a threat increase refuge use.

It is noteworthy that the predator scent treatment showed greater use of cover and low activity levels before the predator was seen. In a study by Chivers *et al.* (2001), slimy sculpin *Cottus cognatus* (Richardson 1836) reacted to olfactory cues regardless of the threat (in this case size) posed by the predator in the absence of other cues, however, when they had visual information they reacted in accordance with the size, and thus danger of the predator. They suggest that chemical cues function as warning of a nearby predator whereas visual cues are needed to provide more detailed information about predator threat. As the olfactory cues were the only information available, prey species have to make assumptions about the threat levels. Potentially, the conditioning may have altered their background behaviour, making them more sensitive and vigilant for dangerous situations. The anti-predator behaviour could be shaped by long term (in this case, days) exposure to predator cues. Likewise, Brown *et al.* (2006) showed that varying background risk was sufficient to cause changes in behavioural response, in accordance with the threat sensitive hypothesis. A threat sensitive reaction may also account for the graded response shown between treatments conditioned using different predator cues. How these responses change is likely to be a function of a variety of factors experienced in the learning phase, such as the frequency of risk (Foam *et al.*, 2005b; Mirza *et al.*, 2006) and type of cue (Chivers *et al.*, 2001; Foam *et al.*, 2005a). Moreover, there is evidence that such negative experiences can have long lasting effects on behaviour. For example, rainbowfish *M. eachamensis* avoid both microhabitats and locations previously associated with predators (Brown, 2003) and retained information about escape routes for almost a year after a brief training period (Brown, 2001). Such long term responses to negative stimuli suggest that the effects of pre-release training may be long lasting. Further understanding of the nuances of learned predator recognition

and anti-predator response would be helpful in making full use of the plasticity of learned predator recognition, such as in pre-release training schemes. Sadly we still lack even basic information about the anti-predator behaviour of Australian native fishes.

The use of Pre-release training has been successfully applied on an ever increasing number of species (Brown and Laland, 2001). However, use on Australian native species, of either commercial or conservation value, is still in its infancy. Pre-release training has taken place in hatcheries around the world, but research has largely focused on Osteophysan species. Similar research performed for the Murray-Darling Basin Authority (Hutchison *et al.*, 2012) on the closely related *M. peelii* used a live predator to condition the hatchery reared fingerlings. They found reduced movement and sparring in conditioned fish in the presence of a predator. These fish also experienced considerably improved survival rates once liberated. Given the extensive numbers of *M. macquariensis* stocked in Eastern Australia, it is logical to make endeavours to improve survival of stocked fingerlings via all means possible. Even small changes in behaviour can have potentially large impact on survival rates. Changes in behaviour and learned predator recognition, such as is seen in this study, could potentially be utilised to improve the ability of hatchery reared *M. macquariensis* fingerlings to recognise and avoid piscivorous predators using pre-release training with the goal of improving post release survival in stocking efforts. Our results show that learned predator recognition in *M. macquariensis* appears to be highly flexible and pre-release training efforts needs to be tailored to the species and the environment into which they are to be liberated for maximum effect. This would require a thorough understanding of the mechanisms at play in order to manipulate it to best suit circumstances, and presents the opportunity for ongoing, species specific research in that area.

6.6 References

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Chapter 7

**Behavioural interactions between conditioned, hatchery-reared trout cod
Machullochella macquariensis and predatory spangled perch *Leiopotherapon
unicolor* in a semi-natural enclosure**

This chapter is formatted for publication in the Journal of Fish Biology. All figures and tables are embedded in text.

7.1 Abstract

Hatchery rearing of fish is known to create behavioural deficiencies when compared with their wild counterparts. One of the ways in which hatchery fish differ is their ability to recognise and react to predators. Life-skills training aims to overcome some of these behavioural differences through behavioural conditioning. In this study, hatchery reared juvenile trout cod (*Machullochella macquariensis*) were conditioned using a combination of damaged released conspecific alarm cues paired with predator cues: visual, olfactory and a combination of both. The fish were then exposed to a live predator under semi-natural conditions. The results show that conditioning trout cod fingerlings enhances their anti-predator response. A graded response was seen in fish conditioned using the different predator cues, with the most intense response observed in those from treatments conditioned with an olfactory component and in particular those featuring combined olfactory and visual cues. These results suggest that pre-release training could be utilised in this species prior to release in stocking programs.

Keywords; trout cod, *Machullochella macquariensis*, hatchery rearing, visual cues, olfactory cues, life skills training

7.2 Introduction

The hatchery environment is different from the natural environment in a number of important respects. Arguably one of the most important differences is the lack of predators in the hatchery environment. It is well understood that predator recognition in fish has a substantial learned component (Kelley and Magurran, 2003), thus without interactions with predators, hatchery reared fish do not fully develop anti-predator behaviours. Data collected internationally suggests a large percentage of released fingerlings do not survive (McNeil, 1991). A lack of well-developed anti-predator behaviour in hatchery reared fish results in significant levels of mortality upon release in the wild (Brown and Laland, 2001; Brown and Day, 2002). Pre-release training aims to overcome some of these deficits through conditioning hatchery reared fish to recognise and react to predators.

One of the ways this is achieved is by conditioning hatchery reared fish with damaged released alarm cues paired with predator cues. When fish are attacked and damaged by predators, the chemicals released into the water illicit innate anti-predator responses in a wide range of species (Brown *et al.*, 2011). When this odour is paired with predator odour, fish associate the two and initiate anti-predator responses on later exposure to the predator odour alone. This conditioning process allows prey to fine tune their predator recognition and anti-predator responses to suit contemporary predator environments. Brown and Smith (1998), for example, showed a single exposure to conspecific alarm cues paired with olfactory cues from a predator could induce increased anti-predator behaviour in hatchery reared trout *Oncorhynchus mykiss* (Walbaum 1792). Moreover, recent work has shown pre-release training can be applied on an industrial scale with large numbers of hatchery fish being successfully conditioned (Olson *et al.*, 2012). This method has proven effective in rapidly training predator naïve hatchery reared fish to recognise predators and their associated cues. Furthermore, it is shown to improve the success of fish post release. D'Anna *et al.* (2012) demonstrated that conditioning hatchery reared sea bream *Diplodus sargus* (L. 1758) to predators and refuge use vastly increased their survival upon release.

The cues used by fish to identify predators are commonly olfactory and visual. The different sensory modalities have properties unique to each that assist prey in identifying and determining the threat posed by a predator. Olfactory cues in predator recognition are widely used in aquatic ecosystems. Olfactory cues spread readily through the aqueous environment and thus can provide information on a potential threat from a large distance. It has been suggested that they act as an early warning (Chivers *et al.*, 2001). For example, coral reef fish *Amphiprion percula* (Lacepède 1802) respond with anti-predator behaviour to olfactory cues but adjust their behaviour when visual cues are also available (Manassa *et al.*, 2013). The addition of visual cues to olfactory cues increases the amount of information available in assessing potential threats. Visual cues have the benefit of being temporally more reliable, providing detailed information about the threat posed by a predator. For example, fish can assess factors like size (Chivers *et al.*, 2001), activity (Brown and Warburton, 1997) and hunger levels (Licht, 1989) of a predator, based on visual cues.

How prey fish choose to use these different cues to recognise predators may be dependent on context. For instance, when visual cues are compromised such as in low light or low water clarity, prey may rely more heavily on olfactory cues, and adjust behaviours accordingly (Hartman and Abrahams, 2000; Leduc *et al.*, 2010). Furthermore, prey fish may choose to rely more on visual cues when undertaking risky behaviours such as predator inspection (Smith and Belk, 2001) or when olfactory cues are ambiguous or unreliable (Brown and Magnavacca, 2003).

The ability to accurately assess the degree of threat posed by a predator is a valuable skill. If prey were to react in a fixed way to all potential predator cues they would lose valuable opportunities to participate in other adaptive behaviours such as foraging. As such, anti-predator behaviour often occurs in a threat sensitive fashion, where the intensity of response corresponds to threat levels (Helfman, 1989). A large and imminent threat is likely to cause prey to cease all other activities and engage in anti-predator responses such as schooling or hiding, while smaller threats may result in intermittent cessation of other activities. By combining the information received by all sensory

modalities, fish can make more accurate assessments of risk. Many studies have found a graded response to varying levels of risk. For instance, McCormick and Manassa (2008) found the response of coral reef fish *Asterropteryx semipunctatus* (Rüppell 1830) to either chemical or visual cues alone was not as strong as the combined effect. Similarly Martin *et al.* (2010) found juvenile roach *Rutilus rutilus* (L. 1758) responded to perceived threat from visual and/or chemical cues in a varied way, highlighting the complexity of such behaviours.

Trout cod *Machullochella macquariensis* (Cuvier 1829) are a large freshwater fish from eastern Australia that inhabits flowing waters with woody debris (Lintermans, 2007). Formerly widespread throughout the southern Murray- Darling system, their decline in abundance has made them a conservation concern. Their previous range extended from the southern tributaries of the Murray- Darling to the Macquarie River (DPI, 2006), their current distribution however, is much more limited. There are indeed, only three self-sustaining populations; Cataract Dam, Sevens Creek and the Murray River between Yarrawonga and Barmah (Lintermans, 2007). Many populations depend entirely upon stocking to replenish numbers and ongoing stocking efforts have led a number of other sites to be close to 'self-sustaining' (Koehn *et al.*, 2013). In fact, *M. macquariensis* are considered a relative success in terms of re-stocking programs (Lyon *et al.*, 2012) and large numbers have been produced in government hatcheries with the number of stocked *M. macquariensis* since 1987 totalling over 1.5 million (Koehn *et al.*, 2013).

In this study the use of predator conditioning in juvenile hatchery reared *M. macquariensis* was examined using both visual and olfactory predator cues paired with olfactory cues of damaged conspecifics. The behaviour of conditioned fingerlings was examined in the presence of a predator at liberty in a semi-natural survival scenario. The ultimate aim of the study was to illustrate the best methods for improving the post-release survival and anti-predator skills of hatchery reared fingerlings.

7.3 Methods

7.3.1 Subjects and housing

Subjects were young of the year *M. macquariensis* of around 5.5cm L_T, reared in outdoor ponds by NSW Industry & Investments, Narrandera. The fingerlings that were used were the first generation offspring of wild caught broodstock. The fingerlings were transported to Macquarie University, Sydney, overnight in oxygenated water packed in Styrofoam boxes. Upon arrival, the fingerlings were transferred into outdoor ponds (capacity 1530L) at a density of 60 fish per pond. The ponds were maintained under bird netting and shade cloth to avoid avian predation. During the first week, the fingerlings were weaned onto flake food using a combination of flake and bloodworms. Thereafter, they were fed flake food 5 days a week and maintained in the outdoor housing ponds until the beginning of experiments.

The fish were then moved into tanks within a laboratory for conditioning prior to testing. The conditioning tanks measured 90cm long x 35cm wide and were filled to 35cm deep. Tanks were placed flush with the wall and all other sides of the tanks (excluding the top) were covered to minimise visual interaction and prevent undue stress on the fish from the activity of lab users. The room had a 12hrs light: 12hrs dark light cycle. Each aquarium was fitted with a filter (capacity 500l/hr), gravel and heater set at 16°C. The fish were placed in these tanks the day before conditioning started, to allow them to settle.

Three wild caught, mature, spangled perch *Leiopotherapon unicolor* (Günther 1859) were used as predators. Spangled perch were chosen as an appropriate species for use as a predator due to their aggressive nature. They are also widespread, with a distribution that overlaps with trout cod. The *L. unicolor* were housed individually in 60 l aquaria, lit overhead by fluorescent lights with a 12 hr light: 12hr dark light cycle. Each aquarium was fitted with a filter (capacity 500l/hr), gravel and heater set at 18°C. They were fed a diet of frozen prawns four times per week. Feeding was suspended during

experimental periods to enhance hunting motivation. The predator's housing tank had no water changes and was not cleaned for a week prior to the conditioning stage. Each individual was not used for more than two sequential trials to maintain motivation. After the conditioning, they were placed back in their housing tank and their feeding resumed.

7.3.2 Conspecific extract (CE)

Conspecific extract (CE) was generated by homogenising whole conspecifics and passing the liquid through a filter (see Chapter 2 Methods 2.3.3.1 for details).

7.3.3 Conditioning

A total of 12 replicate fingerlings were conditioned per treatment. Fingerlings were conditioned to predator cues in groups of 6. Two batches of six fingerlings were conditioned for each treatment. Fingerlings were conditioned in four separate tanks. Each group was designated to one of four treatments (see below for details). Each treatment was exposed 3 times per day to the predator stimuli between the hours of 9am – 10am, noon – 1pm and 3pm – 4pm for 3 days (a total of 9 exposures). This was determined to be a suitable number of exposures after extensive pilot studies. The four treatments were as follows;

7.3.3.1 Predator scent combined with CE

Fish in the predator scent combined with CE treatments (henceforth abbreviated to predator scent treatment) received a combination of conspecific extract and predator scent only during conditioning (ie, no visual cues) (sensu Brown and Smith, 1998). Water that contained the chemical signatures of the predator was used as predator scent. Immediately prior to exposure, 20ml of water was collected from a 60l tank containing a single *L. unicolor* who had been housed in the tank for an extensive period of time. The tank had no water changes or cleaning done for a week prior to the

conditioning stage. The scent was added to the conditioning tank immediately followed by 20ml CE. At the completion of each conditioning the tank was drained completely, cleaned and refilled with de-chlorinated tap water, ready for the next group to be conditioned.

7.3.3.2 Live predator combined with CE

During conditioning, fingerlings from this treatment (henceforth abbreviated to live predator treatment) were exposed to a live predator behind a barrier. A partition made from fine mesh (2mm²) was placed at 1/3 of the length of the tank along, creating two sections; a large section (measurements 62cm x 35cm depth x 35cm wide) and a small section (measurements 31cm x 35cm depth x 35cm wide). The predator was placed in the small section of the tank whilst the fingerlings were placed in the large section. Water was able to pass through the partition, allowing circulation of water and chemical cues. An opaque partition placed adjacent to the mesh was attached to a pulley, and could be lifted remotely. This was lifted at each exposure, for a duration of 15min, to allow visual contact with the predator. This was done in conjunction with the addition of 20ml CE. The predator was removed over night to prevent it attempting to escape. At the completion of each conditioning period, the tank was drained completely, cleaned and refilled with de-chlorinated tap water.

7.3.3.3 Visual cues only combined with CE

During this treatment (henceforth abbreviated to visual cue treatment) received a combination of CE and visual contact with the predator, in the absence of any chemical cues from the predator. This was achieved by keeping the fingerlings in a tank covered with black plastic on three sides to limit any other visual cues. On one short side, a tank of the same dimensions containing a predator was placed flush with the fingerling tank. An opaque Perspex barrier was placed between the two tanks and was attached to a remote pulley that allowed it to be lifted, providing visual contact between the two tanks. At the conditioning times, the predator was gently guided to the end of its tank closest to the fingerlings and contained there by placement of a clear barrier at 30cm away from the

end of the tank. The visual barrier between the two tanks was lifted and the CE carefully added to the fingerlings tank at the end nearest the predator. The barrier remained up for 15min. At the end of the 15min, it was lowered and the predator was released by removing the clear partition. At the completion of each conditioning the fingerlings tank was drained completely, cleaned and refilled with de-chlorinated tap water.

7.3.3.4 Control

This treatment was representative of standard hatchery fish used for stocking and therefore the fish received no conditioning prior to testing. The fingerlings were placed in conditioning tanks of the same dimensions and water volume as the other treatments the day prior to when conditioning would begin. In place of the exposures, the control group were simply left in the aquaria for the equivalent amount of time. Before the next group of fish was conditioned, the tank was drained completely, cleaned and refilled with de-chlorinated tap water.

7.3.4 Experimental Procedure

The testing was performed in a glass tank measuring 245cm x 48cm deep x 61cm tall, this was divided into 2 by a barrier placed 60cm from one end to create a predator zone, where the predator could be released into the testing area with the fingerlings (Fig 7.1). The testing area was marked at every 30cm to indicate the different 'zones'. Zones alternated open – planted from 1 to 6 up to the predator zone. Planted zones were furnished with plants and hides to provide extensive cover for the fingerlings to seek refuge in. Each planted zone contained 1 black, round pot (140mm diameter) with the base cut out and weights glued to the side to sink it, creating a cylinder large enough for the predator to swim through. Each planted zone also contained a small, black, square punnet (50mm at widest), similarly adjusted, that the predator was too large to comfortably fit into. In addition, each planted zone contained 5 large plastic aquarium plants. This combination of items gave dense cover

and ample hiding places. The orientation of each of these items within their planted zone was changed between each trial, so the predator did not become too familiar with the layout. Open zones were left clear of plants and other items. A thin layer of pebbles covered the entire tank to reduce the reflectiveness of the glass base.

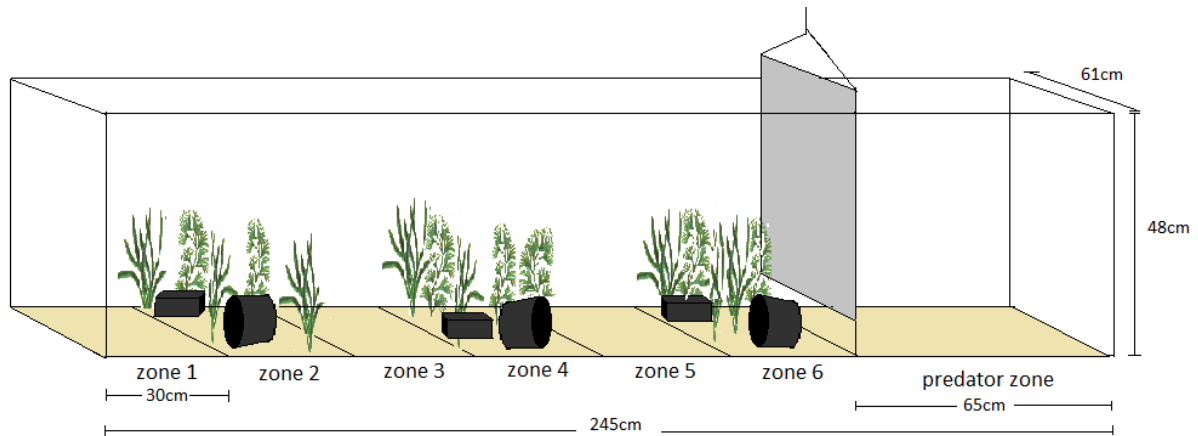


FIG. 7.1 Layout of the experimental tank. Zones 1, 3 and 5 were 'open' zones with no structures and zones 2, 4 and 6 were 'planted' zones, furnished with plants and refuges. The predator zone was separated by a Perspex barrier that could be lifted remotely.

Fingerlings were given 1 hour to settle, before beginning a 20min test. The behaviour of both the fingerling and predator was observed. The use of refuge and open areas (termed area use), activity (measured as the number of borders crossed) and distinct behaviours were recorded. Fingerling behaviours recorded included startle, approach, escape, latency to first movement and failed escape. A startle was defined as a rapid, evasive movement, generally short in distance, away from the predator directed towards escape or avoidance. An approach was any distinct, investigatory movement towards the predator. A failed escape was when the predator caught and killed the fingerling, while an escape was when the predator captured but mishandled the fingerling resulting in its escape. Predator behaviours recorded were strike, approach, capture and failed capture. A strike was a distinct and fast lunge at the fingerling, approach was in this instance the predator approaching the fingerling. Capture refers to the predator capturing the prey and failed capture

refers to the predator attempting but failing to capture prey. Escapes from the fingerlings and failed captures by the predator reflect the same event, as do failed escapes and captures. Ultimately, the numbers of escapes (1) and failed escapes (3) remained too low to include in the analysis.

7.3.5 Data Analysis

Two observers recorded the data during the trial. One observer recorded the behaviour of the fingerling, the behaviour of the predator was recorded simultaneously by the other observer. Data was recorded using the EthoLog program (Ottoni, 2000). Distinct behaviours (approach, startle, escape and failed escape) were too unreliable to be used and thus were excluded from analysis. Predator activity levels were not found to be significant for treatment ($F_{3, 43} = 1.97$, $P = 0.134$). Thus, it can be assumed the levels of motivation from the predators did not differ significantly between treatments. ANOVA and Repeated measures ANOVA were used to analyse the data. The planted data was squared, the number of borders crossed was square root transformed and the latency to move was log transformed to achieve normality. The only exception to this was the data for planted area use, which was normal and thus left untransformed. Data were further scrutinized using a series of post-hoc tests as required. Post-hoc tests used included one-way ANOVA and Fisher's PLSD. All data analysis was conducted using Statview 5.0.1 (SAS Institute Inc.).

7.4 Results

Latency of the fingerlings to move after the addition of a predator in a behavioural interaction trial with a free predator in semi-natural conditions was examined using a one way ANOVA and was found to be strongly affected by treatment ($F_{3, 43} = 10.42$, $P = <0.0001$) (Fig. 7.2). Post hoc tests

indicated the fish from the control treatment had a significantly longer latency to move than those from the predator scent (Fisher's PLSD $F_{3, 43} = 10.42$, $P = <0.0001$) and the live predator (Fisher's PLSD $F_{3, 43} = 10.42$, $P = <0.0001$) treatments. Fish from the visual cue treatment also demonstrated a longer latency to first move than those from the predator scent (Fisher's PLSD $F_{3, 43} = 10.42$, $P = 0.003$) and the live predator (Fisher's PLSD $F_{3, 43} = 10.42$, $P = 0.003$) treatments, although the visual cue treatment fish did not differ statistically from the control treatment.

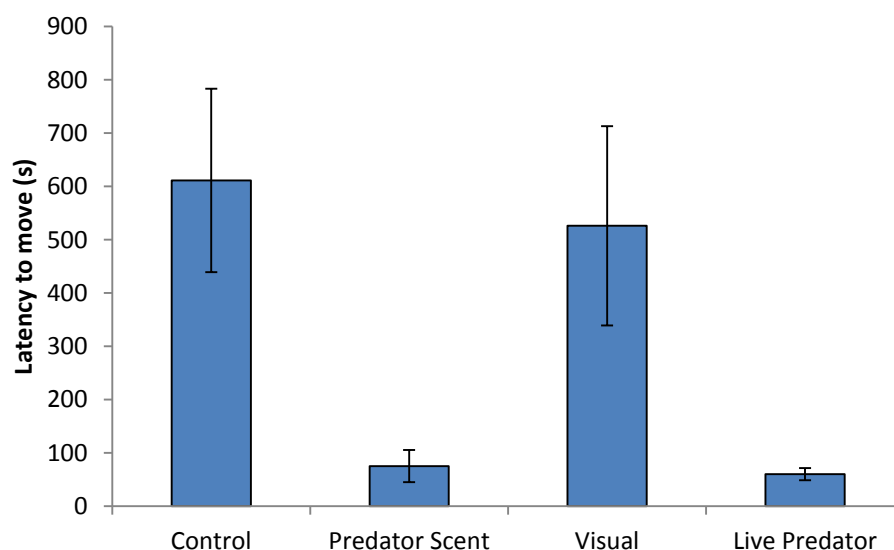


FIG. 7.2 Mean (\pm SE) latency to move of trout cod fingerlings conditioned with one of four treatments; live predator, predator scent, visual cue and control.

The first 20 min of observation of the behavioural interaction trial with a predator at liberty in semi-natural conditions, broken into 5min intervals, were investigated for changes in area use and activity levels. Results of a RM ANOVA showed there was no main effect of treatment (planted; $F_{3, 129} = 1.14$, $P = 0.343$), nor was there an effect of time (planted; $F_{3, 129} = 1.61$, $P = 0.192$). There was, however, a treatment by time interaction for the use of planted areas by conditioned fingerlings (RM ANOVA planted; $F_{9, 129} = 3.19$, $P = 0.002$) (Fig. 3). A significant decrease in use of the planted zone over time

was observed in the fish from the control treatment ($F_{3, 33} = 4.77$, $P = 0.007$). Fish from the predator scent treatment ($F_{3, 30} = 3.36$, $P = 0.032$) and visual cue treatment ($F_{3, 33} = 2.97$, $P = 0.046$) also showed evidence of recovery over time, whilst the fish from the live predator treatment showed no significant change in planted zone use. Further pairwise comparisons showed the fish from the control treatment initially spent more time in the planted zone than fish from the predator scent treatment ($F_{3, 63} = 6.46$, $P = 0.0007$), the live predator treatment ($F_{3, 66} = 4.36$, $P = 0.007$) and the visual cue treatment ($F_{3, 66} = 4.47$, $P = 0.006$) who recovered more slowly.

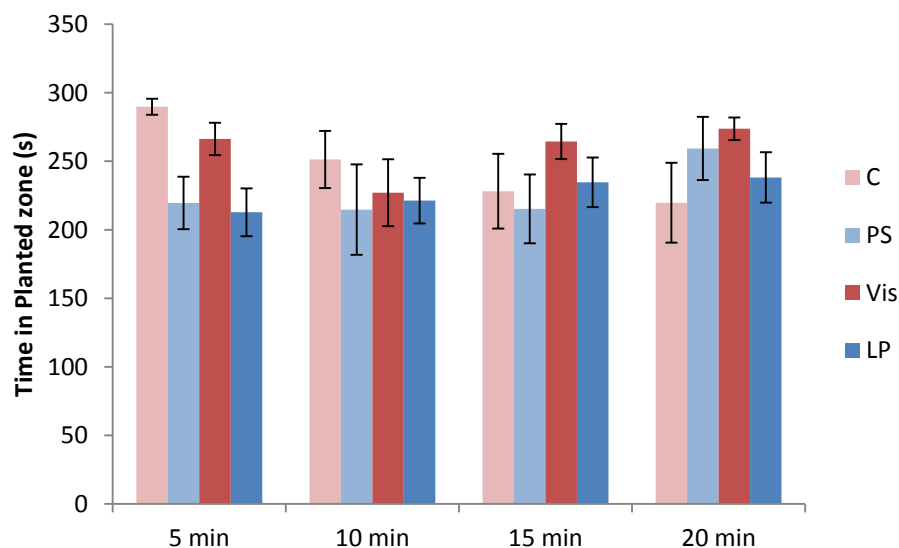


FIG. 7.3. Mean (\pm SE) use of planted zone (s) by conditioned trout cod fingerlings. Fingerlings were conditioned with one of four treatment; predator scent (PS), live predator (LP), visual cue (Vis) and control (C).

The number of borders crossed by fingerlings (activity, Fig. 7.4) was not affected by treatment ($F_{3, 129} = 2.26$, $P = 0.095$) or time ($F_{3, 129} = 1.69$, $P = 0.172$). Activity, however, had a strong treatment by time interaction ($F_{9, 129} = 3.88$, $P = 0.0002$). Fish from both the live predator and the predator scent treatments showed much higher initial activity levels than those from the control treatment (live predator $F_{3, 66} = 7.54$, $P = 0.0002$, predator scent $F_{3, 63} = 6.05$, $P = 0.0011$ respectively). The fish from

the visual cue treatment were also initially less active than those from the live predator and predator scent treatments, however, they did not differ from the control treatment.

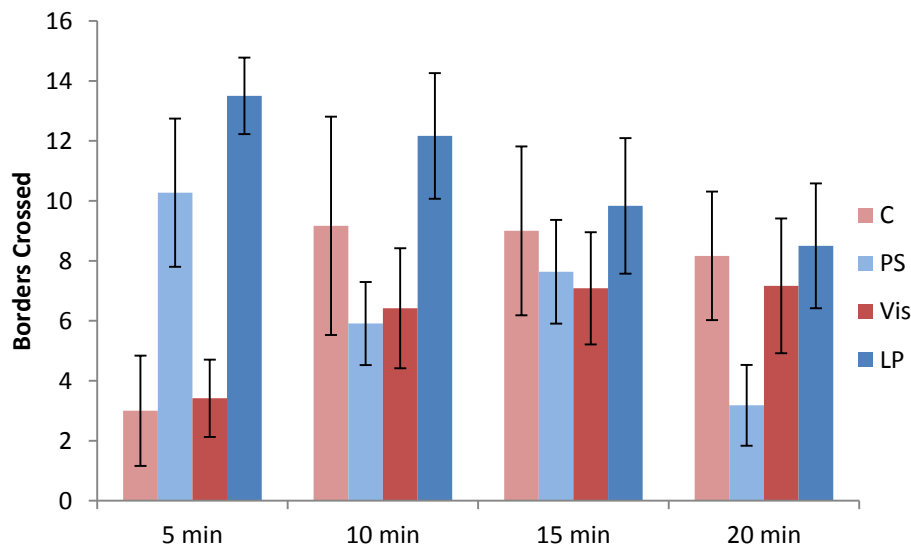


FIG. 7.4. Mean number (\pm SE) of borders crossed (activity) by conditioned fingerlings per 5min of observation.

Fingerlings were conditioned with one of four treatments; live predator (LP), visual cue (Vis), predator scent (PS) and control (C).

7.5 Discussion

Conditioning juvenile hatchery reared *M. macquariensis* fingerlings with predator cues was sufficient to change the way they behaved in the presence of a predator. A graded response was observed between fingerlings in different treatments with those including an olfactory component eliciting the greatest reaction. The fingerlings from the control treatment reduced use of cover as they recovered from the initial exposure to the predator, and differed significantly in their use of cover from fingerlings in all other treatments. As such, it appears all forms of conditioning changed use of cover,

but the fingerlings from the live predator treatment showed the longest effect in increased cover use, followed by fingerlings conditioned using predator scent. The disparity between treatments conditioned using different predator cues suggests that a range of information is acquired through the different sensory modes utilised during conditioning.

The improvement in anti-predator behaviour seen as a result of a learned association of damaged conspecifics and a predator scent cue is consistent with a large body of literature focusing on the role of olfactory cues in learned predator recognition. Early work by Brown and Smith (1998) showed that paired damage released alarm cues and olfactory cues from a predator could successfully condition hatchery reared fish to react aversively to predator cues alone. Since then, a number of other studies have shown the extensive role of olfactory cues in learned predator recognition and in predator- prey interactions (for review see Wisenden, 2000; Ferrari *et al.*, 2010; Brown *et al.*, 2011).

Fingerlings from treatments that were conditioned using olfactory cues (the live predator and the predator scent treatments) were faster to move following exposure to the predator than those conditioned with-out an olfactory component. Fingerlings from the conditioning treatments containing an olfactory component (live predator and predator scent) were also more active generally. Furthermore, the combination of visual information with chemical cues (live predator treatment) did not seem to improve performance significantly over that of olfactory cues alone (predator scent treatment). Likewise, using only visual cues did not appear to facilitate any change in latency of the fingerlings to move following the initial exposure to the predator. Potentially, this is a reflection of a preference for the use of olfactory cues in this species. Such a preference may be context specific and indicative of environmental conditions preferred by the species.

The environmental conditions favoured by *M. macquariensis* often have woody structure (Lintermans, 2007) which could potentially obstruct visual cues. If one cue is unavailable, such as in situations of low visibility, or compromised the other cues may be relied upon more heavily. Leduc *et al.* (2010) described this in Salmon *Salmo salar* (L. 1758), where they responded more strongly to

olfactory cues at night then they did during the day. Likewise, larval newts *Notophthalmus viridescens* (Rafinesque 1820) are better able to discriminate between predatory and non-predatory heterospecifics using chemical cues, not visual, which is likely a result of a habitat with unreliable visual cues (Mathis and Vincent, 2000). Indeed, such sensory compensation may also arise as a result of rearing conditions. Chapman *et al* (2010) found guppies reared in lowlight displayed developmental plasticity which increased their reliance on olfactory cues. Thus, it is possible the greater intensity of response shown by the fingerlings conditioned using olfactory cues was reflective of the environment preferred by *M. macquariensis*.

It appears the fish from different treatments responded with a very graded effect in anti-predator behaviour. Whilst conditioning the fingerlings with olfactory cues only (predator scent treatment) or with both olfactory cues and visual cues (live predator treatment) elicited the most intense response, a small increase in cover use was observed in fingerlings conditioned using only visual cues. A number of studies have found a combined or additive effect with combined visual and olfactory cues. Kim *et al* (2009) found juvenile *S. salar* of different ages differed in the way they used visual and chemical cues, yet both used them in a combined fashion. Likewise, McCormick and Manassa (2008) demonstrated that *A. semipunctatus* use both visual and chemical cues in an additive way to assess risk. Using the combined information of both visual and olfactory cues is likely to give the fingerlings a greater array of information allowing them to assess threat levels accurately. Threat sensitive responses to predator cues have been well documented in fish and it is entirely possible the variety of responses observed from the different treatments was a threat sensitive response to varying degrees of threat during conditioning. Similar patterns of threat sensitive responses have been observed by Holmes and McCormick (2011) who demonstrated that newly settled reef fish *Pomacentrus amboinensis* (Bleeker 1868) presented with varying threat levels of visual and olfactory cues behaved in a way that matched those levels of threat. The level of perceived threat during conditioning may have led to the fingerlings responding to the live predator in a way that corresponded to the level of threat during conditioning. By reacting appropriately to a

potential threat prey are likely to experience increased success and survival. If predator conditioning of hatchery reared fish can improve anti-predator responses it has the potential to improve their post-release survival.

International studies have shown that stocking programs are often unsuccessful and that hatchery reared fish suffer high levels of mortality owing in part to behavioural deficits (Olla *et al.*, 1998; Brown and Laland, 2001). Furthermore, there is evidence to suggest that there are no long term improvements in catch for stocked rivers (Young, 2013). Population modelling by Rogers *et al.* (2010) suggested that stocking can improve fisheries of the closely related Murray cod *Machullochella peellii* (Mitchell 1838) in areas where there is low natural recruitment or overfishing. Indeed, the reintroduction of *M. macquariensis* into some areas has been considered a relative success (Koehn *et al.*, 2013). Scientists funded by the Murray-Darling Basin Authority (Hutchison *et al.*, 2012) showed predator conditioning of *M. peellii* improved anti-predator behaviour and furthermore, vastly improved the survival of hatchery reared juveniles after release. The stocking report for DEPI Vic for (DEPI, 2013) stated a release of over 4000 *M. macquariensis*. Given the significant investment required to do this and supporting evidence from the closely related *M. peellii*, it would be prudent to make efforts to improve stocking success of *M. macquariensis* by incorporating some form of pre-release training. The results presented herein highlight the plastic nature of behaviour and the role of experience with predators in learning anti-predator skills. By understanding the details specific to the species, a tailored approach can be constructed to achieve the best outcome. Furthermore, the results support the possibility of predator conditioning being a feasible procedure to improve the post-release survival and anti-predator behaviour of hatchery reared *M. macquariensis*.

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Chapter 8

General Discussion and Conclusions

8.1 General Outcomes

When the preceding chapters are considered together the range and variation of behaviours between species and between treatments within a species becomes evident. However, no one overwhelming pattern of behaviour across the species is apparent. This is an interesting result and serves to highlight the point that behavioural traits are unique to each species. Furthermore, in the context of life-skills training, it reminds us that each species needs to be addressed individually in order to create the most effective and beneficial conditioning program.

The general outcomes observed in each species were as follows:

Chapter 2; Predator conditioning in hatchery reared golden perch *Macquaria ambigua* : The roles of visual and olfactory cues in learned predator recognition – A graded range of responses were observed across all treatments with fingerlings trained using a combination of olfactory cues and CE reacting the most intensely. Conditioning using only CE elicited a response that was more generalised than that of the fingerlings conditioned with predator scent and CE, but similar in intensity.

Chapter 3; Behavioural interactions between conditioned, hatchery-reared golden perch *Macquaria ambigua* and predatory spangled perch *Leiopotherapon unicolor* in a semi-natural enclosure - A range of responses were seen from fish trained with the different cues, with multiple cues eliciting the most intense response. Conditioning the fingerlings with both olfactory and visual cues resulted in an increased use of cover and a trend towards fewer approaches toward the predator.

Chapter 4; Predator conditioning in hatchery reared Australian bass *Macquaria novemaculeata*: The roles of visual and olfactory predator cues in learned predator recognition – The differences in behaviour observed depended not only on the treatment the fingerlings were exposed to but the tests of their anti-predator behaviour. When examining area use fingerlings conditioned using only scent cues were the most sensitive, making use of refuge. They also tended to school more loosely. Fingerlings conditioned using visual cues or multiple cues tended to recover more quickly. Previous experience appeared to play a large role in how the fingerlings behaved and generally, experience with visual cues resulted in a less timid response to the predator. The test itself also revealed clues about the threat sensitive nature of the behaviours, as schooling responses were less intense when fingerlings were presented with olfactory cues rather than visual cues which were presumably indicative of a greater level of threat.

Chapter 5; Behavioural interactions between conditioned, hatchery-reared Australian bass *Macquaria novemaculeata* and predatory spangled perch *Leiopotherapon unicolor* in a semi-natural enclosure -Using multiple cues during conditioning (live predator) resulted in the strongest response from the fingerlings, with a graded response observed from the other treatments. A trend towards a greater reliance on visual cues was also observed. Movement, rather than use of refuge in the planted areas was in this instance the more sensitive behavioural assay.

Chapter 6; Predator conditioning in hatchery reared trout cod *Machullochella maquariensis*: The roles of visual and olfactory cues in learned predator recognition– Conditioning fingerlings using multiple cues (live predator) induced the greatest range of response, including predator inspection. A graded response was seen from the fingerlings conditioned using the other treatments, which was most likely threat sensitive in nature.

Chapter 7; Behavioural interactions between conditioned, hatchery-reared trout cod *Machullochella maquariensis* and predatory spangled perch *Leiopotherapon unicolor* in a semi-natural enclosure - Conditioning the fingerlings using multiple cues (live predator) or cues containing an olfactory

component from the predator (predator scent) resulted in fingerlings that were faster to first move. These fingerlings were also more active and made less use of refuge in the planted areas.

8.2 Behavioural responses to conditioning

All species appeared to adhere to a threat sensitive response (Helfman, 1989) and in doing so are likely to experience an adaptive benefit from the training regime once released into the wild. This is a widespread response to varying levels of threat and thus it is not surprising that they all exhibited a graded response to the different conditioning treatments. How this can be manipulated in a life skills training scenario would be an area of research to continue investigating. By training using high threat levels you could conceivably create fish that react in an intensely vigilant manner, however, this may need to be balanced against possible habituation to the cues after repeated exposures (Berejikian *et al.*, 2003; Vilhunen, 2006). Evidence suggests that much of the post release mortality from predation occurs very shortly after liberation (Olla *et al.*, 1998; Brown and Day, 2002), thus such induced vigilance need only persist for this short period while the fish learn more temporally relevant predator recognition skills and anti-predator behaviour *in situ*.

Another notable feature that is common amongst all three species is the flexibility and behavioural plasticity exhibited. Predator recognition involves a large learned component, as is well documented (Brown *et al.*, 2011) and this allows prey fish to develop a behavioural repertoire that is tailored to very specific contemporary environmental conditions. As with a threat sensitive response, having a flexible and plastic learning ability is likely to confer an adaptive benefit and thus is a common feature amongst all three species. These behavioural features give restocking programs great scope to manipulate the behaviour to suit conditions into which the fish are to be stocked. By having a thorough understanding of the behavioural response of a stocked species, along with an appreciation for specific challenges and threats that they may face upon liberation, a custom-made training regime can be designed. This is only possible due to the highly flexible nature of learned predator recognition and anti-predator behaviours.

Generally speaking, in each of the species studied in this research project, the use of multiple predator cues (live predator treatment) when paired with CE during conditioning was one of the stronger, if not the strongest, enhancer of anti-predator behaviour. This suggests a reinforcing effect that is likely gained from the greater degree of information gathered through using both sensory modalities. Conditioning using only a single sensory modality, however, gave a better indication of the behavioural tendencies unique to each species. Potentially, any similarities in behaviour pattern noted between these species are a consequence of similar environmental preferences. For instance, during the behavioural interaction trial with a predator at liberty in semi-natural conditions, trout cod *Machullochella maquariensis* conditioned using olfactory cues had a much more dramatic response than the control or visual treatments. Likewise the Golden perch *Macquaria ambigua* also exhibited a tendency to rely on olfactory cues during the predator conditioning phase. The habitats preferred by *M. ambigua* are deep water (Crook *et al.*, 2001), turbid lowland rivers with woody structures (Lintermans, 2007). Similarly, the habitats of *M. maquariensis* are structured (Nicol *et al.*, 2007) and they are usually associated with a snag or woody habitat (Grown *et al.*, 2004). The pattern of behaviour seen in the experiments presented herein may result from a similarity in habitats that make olfactory cues the more reliable sensory modality, as both *M. maquariensis* and *M. ambigua* are commonly associated with woody snags and structures. However, beyond that, the general behavioural differences between the species make it difficult to compare them directly to one another.

8.3 Outcomes and their consequences

The results presented herein contribute to a growing body of literature focusing on predator-prey interactions. They also contribute to the general understanding and knowledge of each of the individual species focussed upon. Behavioural studies focusing on Australian freshwater fish are limited and mostly focus on small species such as rainbowfish (for instance Brown and Warburton, 1997; Brown, 2002; 2003; Keller and Brown, 2008; Kydd and Brown, 2009). Studies examining the

behaviour of larger, often predatory fish are notably lacking. This may be due to the logistical difficulties of studying these species, however, it does not mean they are less worthy of the effort. Sadly, there is still little known about the basic behaviour of these species during predator-prey interactions. The results described here have contributed substantially to advancing the understanding of how these species interact and learn about piscivorous predators. How the behavioural traits of each species can be manipulated to improve stocking success and survival of stocked fish after release is of particular interest and this study contributes valuable material towards this end. Furthermore, this research has advanced our understanding of how life skills training can be most effectively applied in these species and has given evidence for the potential for its success. Early work on life skills training in Australian native freshwater species has been undertaken by researchers for the Murray-Darling Basin Authority (Hutchison *et al.*, 2012). Their efforts provided an excellent demonstration of the potential for these practices to be used on native species. Not only did they establish the capacity for behaviours to be changed and remediated, they also validated that this can equate to improved survival of conditioned fingerlings (Murray cod *Machullochella peelii*) post-release. The value of such study is clearly indicated by such success and advocates continued efforts into this field of research.

Internationally, the study of life skills training has been increasing for many years and is noted as a useful tool in improving behaviour of hatchery reared fish in many locations. The effectiveness of life skills training has been noted in flatfish *Paralichthys sp* from Japan, (Kellison *et al.*, 2000; Hossain *et al.*, 2002), Arctic charr *Salvelinus alpinus* in Scandinavia (Vilhunen, 2005; Vilhunen, 2006), Chinook salmon *Oncorhynchus tshawytscha* in the USA (Berejikian *et al.*, 2003), rainbow trout *Oncorhynchus mykiss* in Canada (Brown and Smith, 1998) and Atlantic Salmon *Salmo salar* in the UK (Brown *et al.*, 2003) to name a few popular examples. The use of life skills training also extends beyond fish to other species such as aquaculture reared invertebrates (Davis *et al.*, 2004; Davis *et al.*, 2005). The investigation of this technique is widespread and it is a worldwide movement that has been slow to

be adopted in Australia and there is still a huge gap between fisheries practices and those used on other vertebrates (Brown and Day, 2002).

Australian native fish reared in hatcheries are generally produced for either fisheries enhancement or for conservation purposes. Vast amounts of time and money are injected into these stocking programs and logic would dictate that all efforts are made to make such programs as efficient and successful as possible and that the success and ongoing survival of the stocked fish is monitored. In both conservation and fisheries enhancement the efficacy and success of a program is contingent on the hatchery stock surviving and growing to a size where they either reproduce or recruit into the fishery. The need for ongoing assessments and following up on the survival statistics of released hatchery fish should be emphasised. Knowing if a program has been successful or not, and where it failed will help to make future efforts more effectual. As such, any action that results in improvement of survival is worthy of investigation. This may be simple acts like increasing environmental complexity during rearing (Brown *et al.*, 2003) through to highly tailored behavioural remediation programs. The conditioning approach used may be tailored to suit a specific environment or problem. For instance, if the fish are to be released into very structured habitats shelter conditioning may better prepare the fish for liberation (Kawabata *et al.*, 2011). Furthermore, recent research has shown fish can begin the process of learned predator recognition in embryonic stages (Nelson *et al.*, 2013; Oulton *et al.*, 2013). This provides an attractive option for conditioning large groups of fish early in their production which is worth considering. Further research efforts aimed at improving efficacy of conditioning programs may include topics such as investigating behavioural details of individual species, thus allowing a tailored conditioning programs that is specific for both species and environment into which they are released, and economical methods of conditioning that can be easily applied.

A number of aspects require thought when considering the use of life skills training in hatchery reared fish in Australia. Firstly, and most importantly, is that such programs should not occur in

isolation. It is important that a holistic approach is considered and that other threatening processes to the species are identified and addressed. The threatening processes that are likely to impact on native fish populations have been identified and outlined by the Native Fish Strategy for the Murray–Darling Basin, which proposes a long term rehabilitation for native fish populations (Koehn and Lintermans, 2012). The panel of experts intend an integrated approach to improving fish stocks and their environments, covering many aspects such as water flow regulation, habitat improvement, alien species control through to community involvement. A number of achievements have already been made in this area. Re-snagging, for instance, has been applied to help restore habitats and the utilisation of these habitats by native fish in the Murray River has been noted (Nicol *et al.*, 2004). Likewise, environmental water allocations have enhanced native fish spawning and recruitment, along with other ecological benefits (King *et al.*, 2010). The monitoring of stocked fish is still very limited, however, the capacity to monitor, and distinguish hatchery from wild fish does exist (Crook *et al* 2009, 2011, Woodcock *et al* 2011). As such, the application of monitoring programs is highly feasible. Whilst many achievements have been made in the improvement of fisheries of the Murray-Darling, much work remains to be done, which is likely to encompass a number of complicated issues and require a great deal of ongoing research.

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ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2009/046-4

Date of Expiry: 15 June 2013

Full Approval Duration: 16 December 2009 to 15 June 2013 (36 months + extension)

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) and will only be renewed upon receipt of a satisfactory Progress Report before expiry / is contingent upon receipt of a Final Report at the end of this period (see Approval email for submission details).

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In case of emergency, please contact:
the Principal Investigator / Associate Investigator named above,
Animal Welfare Officer - 9850 7758 / 0439 497 383, or Manager, Fauna Park - 9850 4109 / 0425 213 420

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: Life skills training in hatchery reared fish

Type of animal research – 4 – Research - Human or Animal Biology

Aims of the project:

This project aims to investigate the acquisition of learned predator recognition and foraging skills in fish that have been bred and reared in captivity. Fish are split into groups of roughly 25 and housed in ponds; fish undergo: 3 weeks acclimatization; 1 month treatment and conditioning; 1 week testing; 2 weeks final conditioning; survivorship test; 1 month housing in semi-natural pond; weighing; euthanased at completion. During conditioning and testing, fish are exposed to a live (but restrained) predator, and alarm cues are collected by homogenising the tissue of 2 fingerlings per species that have been euthanased via MS222/sodium bicarbonate.

Procedure: 3 Minor conscious intervention

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Total
350 per study species (Australian Bass, Golden Perch, Trout Cod, Eastern Cod) and 2 of each predator species (Murray Cod, Spangled Perch)	1404
50 Australian Bass, 50 Golden Perch	100
Total	1504

Location of research:

Location	Full street address
Fauna Park	Macquarie University NSW 2109
Central Animal Facility	(Rooms 159,127 & 128) Macquarie University NSW 2109

Amendments approved by the AEC since initial approval:

1. A copy of the permit from NSW Fisheries to be provided to the Animal Welfare Officer upon receipt (Received Nov 2010).
2. Extension to approval duration - 6 months and additional fish (Approved AEC December 2012)

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.



Prof Michael Gillings (Chair, Animal Ethics Committee)

Approval Date: 6 December 2012