Colour Change and Camouflage in Rockpool Fish

Volume 1 of 1

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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Abstract

Camouflage is one of the most widespread anti-predator strategies in nature. Many animals use a combination of both morphological and behavioural means. Camouflage can be particularly challenging in heterogeneous environments, and as such some animals have evolved under selection to change colour to enable them to camouflage on a range of different background types. One such species is the rock goby (*Gobius paganellus*), a common rockpool fish capable of rapidly (within one minute) changing its colour and luminance (perceived lightness) when placed on different backgrounds. The rock goby provides a good model for studying rapid colour change in fish inhabiting habitats such as rocky shores that tend to be highly heterogeneous, and where fish may be exposed to both terrestrial and marine predators depending on tidal level. I used digital image analysis and a model of predator vision to quantify changes in colour, luminance, pattern, and camouflage. In chapter 2 I investigate the ability of rock gobies to match the colour of sand and algae covered rock, and test whether a fish's previous background affects their ability to match a new one. I also tested their ability to match a range of different background brightness. Finally, I ask whether rock gobies exhibit behavioural background matching in addition to adaptive colour change. In chapter 3 I ask whether rock gobies change their body pattern in response to their visual background, and then whether the spatial frequency of more natural backgrounds influences pattern change. I found that the gobies rapidly changed colour and luminance in response to the different backgrounds and an individual's previous background had no effect on its ability to change colour and camouflage on a new background. The level of camouflage did however differ between backgrounds whereby some colours and brightness appeared easier to match than others. Rock gobies also showed a behavioural preference for darker backgrounds over lighter ones. Moreover, gobies are capable of rapidly changing their body pattern in response to their background, with high spatial frequency substrates such as sand inducing the greatest change in pattern. This thesis shows that small rockpool fish use a combination of rapid colour and pattern change, and behaviour background choice, to camouflage themselves against their background.

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Chapter 1: Overall introduction

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Camouflage and colour change in animals

Colour change has evolved in many different taxa including, reptiles (e.g. Stuart-Fox and Moussalli, 2008; Stuart-Fox et al., 2008), amphibians (e.g. Camargo et al., 1999; Garcia and Sih, 2003), fish (e.g. Sumner, 1911; Ramachandran et al., 1996; Stevens et al., 2014a; Allen et al., 2015), crustaceans (e.g. Stevens et al., 2013, 2014c)), and cephalopods (e.g. Hanlon and Messenger, 1988). Whilst colour change serves many different functions in different animals, this thesis will focus on the use of rapid (occurring in seconds or minutes) morphological colour change for camouflage.

The functions of colour change in animals

The ability of an animal to change the colour and lightness of its body serves different, but not necessarily mutually exclusive, functions in different species; namely thermoregulation, communication, and camouflage (Stuart-Fox and Moussalli, 2009). Colour change has evolved as a means of thermoregulation in a variety of taxa (e.g. Key and Day, 1954; Fernandez and Bagnara, 1991; Castrucci et al., 1997; Silbiger and Munguia, 2008). For example, the fiddler crab *Uca pugilator* responds to changes in environmental temperature by becoming lighter at warm temperatures and darker at cold temperatures (Silbiger and Munguia, 2008). A somewhat similar response is also seen in *Rana chiricahuensis*, a species of leopard frog, which becomes darker at low temperatures (Fernandez and Bagnara, 1991).

In other animals colour change functions for social signalling (Stuart-Fox and Moussalli, 2009). For instance, chameleons change their colour pattern to communicate with conspecifics, and selection for social signalling is thought to be the primary driver behind the evolution of colour change in this group (Stuart-Fox and Moussalli, 2008). The use of colour and pattern change for communication is most predominant in males, which use it to signal their dominance status and to court females (Stuart-Fox and Moussalli, 2008). A dominant male will display colour patterns which are highly conspicuous and contrasting to the background, while submissive males that have lost a contest or been aggressively rejected by a female will display dull, low contrast markings (Stuart-Fox and Moussalli, 2008). In all cases the dominant or submissive coloration displayed by males is species-specific (Stuart-Fox and Moussalli, 2008). The use of highly contrasting signals does however carry costs as

being conspicuous often results in a much greater predation risk (Husak et al., 2006; Stuart-Fox et al., 2003).

The use of colour change for social signalling has also been reported in some anuran species such as the toad *Bufo luetkenii* (Doucet and Mennill, 2010). *Bufo luetkenii* is an explosive breeding species that forms large aggregations during the breeding season. During the breeding season unpaired males are bright yellow but this coloration rapidly changes to a cryptic brown once a male has paired with a mate (Doucet and Mennill, 2010). While it is clear that *B. luetkenii* is changing colour to produce a social signal is it is unknown whether the bright yellow coloration of the males is a signal to attract females or deter rival males (Doucet and Mennill, 2010).

Colour change is particularly prevalent in aquatic environments, and there are multiple examples of it being used for communication in aquatic species (Sköld et al., 2013). Atlantic salmon (*Salmo salar*) involved in territorial contests communicate submission to their opponent by darkening the colour of their skin (O'Connor et al., 1999). In another example, cuttlefish, which are well known for their dynamic colour changing ability (Hanlon and Messenger, 1988; Hanlon, 2007), have been observed to use colour change for signalling not only conspecifics (Zylinski et al., 2011) but also potential predators (Langridge et al., 2007).

The use of colour change for signalling often involves an animal making itself highly conspicuous against its background, whereas in other species or contexts colour change is used for the opposite function: to reduce their conspicuousness through camouflage. Moreover, colour change plays a dual role in many species, whereby it allows an animal to produce bright, high contrast colour patterns for signalling, while allowing them to rapidly change colour for camouflage if threatened or when resting (Hanlon et al., 2007; Stuart-Fox and Moussalli, 2009; Zylinski et al., 2011). The use of colour change for camouflage is widespread among animals (Stuart-Fox and Moussalli, 2011) and will be reviewed in the next section as colour change for camouflage is the focus of my thesis.

Camouflage in colour changing animals

Camouflage is one the most widespread anti-predator strategies in nature (Cott, 1940; Stevens and Merilaita, 2009a; Thayer, 1909). Camouflage functions by preventing detection or recognition by a receiver when the animal is in plain sight, most often through the involvement of body coloration (Ruxton et al., 2004; Stevens and Merilaita, 2009a). Strategies which primarily prevent detection are collectively referred to as crypsis and differ from those such as masquerade, which prevent recognition following initial detection (Skelhorn et al., 2010; Stevens and Merilaita, 2009a). The term crypsis encompasses a number of different camouflage strategies including, but not limited to, background matching (e.g. Endler, 1984), disruptive coloration (e.g. Cuthill et al., 2005), and countershading (e.g. Rowland et al., 2008). In Table 1.1 I define the different types of visual camouflage most important to this thesis.

Although crypsis provides benefits by impeding detection by predators, in many cryptic species that have a fixed coloration it can impose costs by limiting an animals capacity to travel over a range of backgrounds in heterogeneous habitats (Ruxton et al., 2004). The evolution of colour change goes a long way to reducing the cost of crypsis in terms of missed opportunities, by allowing animals to travel across different backgrounds without greatly increasing the risk of detection. Crypsis can be achieved through both morphological and behavioural means, both of which are discussed below in the context of colour changing animals.

Table 1.1: Definitions of the different visual camouflage strategies. All definitions are based on those given in Stevens and Merilaita (2009a) and examples of the primarily literature are also cited as evidence for each camouflage strategy.

Strategy	Definition of function	Evidence in primary literature
Crypsis:	primarily prevents initial detection	
Background matching:	when an animal's appearance generally matches the colour, lightness and pattern of one (specialist) or several (compromise) background types	Endler (1984), and Merilaita and Lind (2005)
Disruptive coloration:	hinders detection or recognition of an animal's, or part of an animal's, true outline and shape by creating the appearance of false edges and boundaries	Cuthill et al. (2005)
Countershading:	when an animal's pigmentation cancels out the creation of shadows by being darker on the side facing the direction of illumination (self-shadow concealment), destroys the shadow/light cues which give away the three-dimensional form of an animal (obliterative shading), or simultaneously matches the lightness of two different backgrounds depending on which direction the animal is viewed from (form of background matching)	Rowland et al. (2007, 2008)
Distractive marking:	direct the 'attention' or gaze of the receiver away from features (such as the outline) which would give away the animal	Dimitrova et al. (2009)
Flicker-fusion:	where markings such as stripes blur during motion to match the colour/lightness of the general background in order to prevent detection when the animal is moving	Lindell and Forsman (1996)
Motion dazzle:	where markings make it difficult for the receiver to make estimates of speed and trajectory	Stevens et al. (2008) and Hughes et al. (2014)
Masquerade:	prevents recognition by resembling an uninteresting or neutral object such as a stick or leaf	Skelhorn et al. (2010)
Motion camouflage:	when an animal moves in a fashion that decreases the probability of a receiver detecting movement	Mizutani et al. (2003)

Crypsis through colour change

Colour change has been used by animals to utilise many of the different types of crypsis highlighted in Table 1.1. For instance the Nassau grouper (*Epinephelus striatus*) is able to alternate between three basic body patterns, barred, mottle, and white belly (Watson et al., 2014). The barred pattern is marked by large, highly contrasting vertical bars on the dorsal and ventral surface of the body (Watson et al., 2014). The authors suggest that these bars have characteristics of disruptive coloration and may hinder detection or recognition by hiding the outline of the fish and creating false edges (Cuthill et al., 2005; Watson et al., 2014). In another example, a series of experiments on three species of cuttlefish, *Sepia officinalis*, *Loligo vulgaris*, and *Octopus vulgari*, demonstrated that they exhibit a 'countershading reflex' when rolled or pitched whereby the upper part of the body is always darker than the lower side (Ferguson and Messenger, 1991; Ferguson et al., 1994).

However, by far the most common (or at least the most studied) form of colour change for crypsis is via background matching (e.g. Sumner, 1911; Hanlon and Messenger, 1988; Ramachandran et al., 1996; Camargo et al., 1999; Stuart-Fox et al., 2008; Clarke and Schluter, 2011; Sköld et al., 2013; Stevens et al., 2014a, 2014c; Allen et al., 2015). This is particularly well studied in flatfish which are well known for their ability to change their colour and pattern to match their background (e.g. Lanzing, 1977; Ramachandran et al., 1996; Burton, 1998, 2002, 2010). In the classic study by Ramachandran et al. (1996), eyed flounders (*Bothus ocellatus*) were able to change their colour pattern in just 2-8 seconds when placed on both artificial checkerboard backgrounds and natural substrates. Pattern and colour change has also been documented in a number of other flatfish species including, but not limited to, plaice (*Pleuronectes platessa*) (Kelman et al., 2006), English sole (*Parophrys vetulus*), northern rock sole (*Lepidopsetta polyxystra*) and Pacific halibut (*Hippoglossus stenolepis*) (Ryer et al., 2008), and turbot (*Scophthalmus maximus*) (Lanzing, 1977).

Flatfish are not the only teleosts which change colour to match their background. In a recent study, Allen et al. (2015) found that slender filefish (*Monacanthus tuckeri*) can change their coloration and pattern within 1-3 seconds allowing them to effectively match their background with the aid of dermal flabs that help to break up the fish's outline. In a different study Clarke and Schluter (2011) investigated the ability of two sympatric species of threespine sticklebacks, a limnetic species which occurs in the pelagic zone, and a benthic

species which inhabits the littoral zone, to alter their dorsal body coloration when exposed to extremes of the two habitat background colours. Both species were able to change their dorsal body coloration to better match the colour of either the pelagic or the littoral backgrounds. Interestingly, the benthic species was better than the limnetic species at matching the colour of the littoral background but there was no difference between the two species regarding their ability to match the pelagic background (Clarke and Schluter, 2011). The authors suggest that the more dynamic colour changing ability of the benthic species was the result of the greater spectral heterogeneity of the littoral zone in which they inhabit (Clarke and Schluter, 2011).

Habit heterogeneity is likely to be a key driver behind the evolution of colour change for camouflage and colour change is likely to be widespread among animal occurring in highly heterogeneous habitats such as rocky shores (Fries, 1942; Keeble and Gamble, 1899; Stevens et al., 2014a, 2014c). Stevens et al. (2014a) tested the ability of a common rockpool fish to change colour for camouflage. When placed on a black or white background, rock gobies (*Gobius paganellus*) responded by turning darker or lighter respectively. Furthermore, rock gobies become redder when placed on a red background, though interestingly they did not turn bluer when placed on a blue background, but instead became greyer thus demonstrating that colour change is not unbounded and that certain colours may be more difficult to match than others (Stevens et al., 2014a).

Numerous other taxa are also able to alter the coloration of their body for camouflage. Shore crabs (*Carcinus maenas*), another common rockpool species throughout the world, are capable of changing their brightness and colour when exposed to different backgrounds (Stevens et al., 2014c). Some species of crab also respond to changes in light level by showing a circadian rhythm of colour change whereby they become lighter during the day and darker at night (Stevens et al., 2013). Among terrestrial species chameleons are well known for their ability to change colour. Although camouflage is not the primary driver behind the evolution of colour change in chameleons (Stuart-Fox and Moussalli, 2008) it is still an important factor influencing colour change in this group. For instance, two species of dwarf chameleons, *Bradypodion transvaalense* and *Bradypodion taeniabronchum*, have been shown to use colour change for camouflage in the presence of predators and that the level of camouflage is predator-specific whereby they show different colour responses to snake and bird predators (Stuart-Fox et al., 2006, 2008).

One of the most studied and well known examples of rapid colour change in animals is cuttlefish. Cuttlefish are able to change not only the colour and pattern of their skin (Hanlon and Messenger, 1988) but also the textural surface of their skin thus breaking up the visual outline of their body, and even allowing some species to potentially masquerade as vegetation (Allen et al., 2009). Evidence of the effectiveness of cuttlefish camouflage is presented in Hanlon and Messenger (1988). Hanlon and Messenger (1988) released 32 lab reared *Sepia officinalis* (aged from under 1 week old to 17 weeks old) into their natural habitat and observed each individual for over an hour. *Sepia officinalis* live in a heterogeneous habitat containing a variety of different backgrounds such as sand, rock and algae (Hanlon and Messenger, 1988). All of the cuttlefish released, regardless of their age, were able to adapt to every substrate they encountered. The authors note that on several occasions after looking away momentarily it was almost impossible to locate the cuttlefish again. More importantly, passing fish (including predatory species) repeatedly failed to detect motionless cuttlefish. *Serranus cabrilla*, a natural predator of *S. officinalis*, remained completely oblivious to the presence of a motionless cuttlefish on over 40 observations. Cuttlefish were only detected by *S. cabrilla* as a result of movement (e.g. swimming or burying) (Hanlon and Messenger, 1988). In their paper Hanlon and Messenger (1988) argued that cuttlefish conceal themselves against different backgrounds either by background matching or via what they referred to as 'disruptive coloration'.

All camouflage body patterns in cuttlefish are widely considered to fall into three main categories, those of uniform, mottle and 'disruptive', see Figure 1.1, (Barbosa et al., 2007, 2008b; Chiao and Hanlon, 2001a; Hanlon, 2007; Hanlon et al., 2009; Kelman et al., 2007; Mäthger et al., 2007, 2008; Zylinski et al., 2009). Uniform body patterns have little or no variation in contrast across the body. Colour and brightness can vary between different uniform patterns, but within any single pattern both colour and brightness are constant across the entire body (Hanlon and Messenger, 1988; Hanlon et al., 2009). Stipple patterns, which differ from purely uniform ones in that they have many small dark spots resulting from small clusters of expanded chromatophores, are categorised under uniform and are characteristic of a transition phase between uniform and mottle patterns (Hanlon and Messenger, 1988; Hanlon et al., 2009). These dark spots are evenly distributed across the body and bear a close resemblance to fine substrates such as sand. In nature, uniformly coloured sand, mud and rock tend to elicit a uniform or stripple body pattern (Hanlon, 2007; Hanlon et al., 2009). In contrast, mottle body patterns consist of fairly evenly distributed small-to-moderate-scale

light and dark patches (sometimes referred to as mottles) which are coarse-grained in appearance. The size and shape of the light and dark patches and their constants between one another varies depending on the background being matched (Hanlon and Messenger, 1988; Hanlon, 2007; Hanlon et al., 2009). Lastly, the so-called 'disruptive' body patterns are characterised (at least in cephalopods) by highly contrasting light and dark patches which vary in shape, size and orientation (Hanlon and Messenger, 1988; Hanlon, 2007).

Visual features affecting background matching- examples in cuttlefish

A great deal of research has been carried out over the last two or so decades aimed at identifying the different visual stimuli that elicit each of the different camouflage strategies described above. Although much is still unknown, we now have a good understanding of how cuttlefish choose the best camouflage pattern based on their perception and interpretation of the world around them. Much of our current knowledge is based on the findings of lab experiments which used checkerboard patterns to investigate how cuttlefish respond to different visual backgrounds. One such study by Chiao and Hanlon (2001a) tested six lab reared *Sepia pharaonis* on a series of computer generated checkerboards. In each trial the cuttlefish were recorded every 2 seconds for 30 minutes using a digital video camera. A human subjective grading scheme of patterning was used to quantify the responses of the cuttlefish to the different checkerboard backgrounds. In their first experiment Chiao and Hanlon (2001a) tested different checker sizes to determine how the size of the substrate elements influenced the cuttlefish's choice of camouflage. The checkers ranged in size from approximately 13% to 130% of the size of the cuttlefish's 'white square' component. The 'white square' is a rectangular area located in the centre of the dorsal mantle of cuttlefish (see Figure 1.1). It is most apparent in disruptive body patterns and is highly contrasting to the surrounding dark components (Hanlon & Messenger 1988). The second experiment used checkerboards that were all equal in check size but varied in the percentage contrast between the dark and light squares. A third experiment looked at how the number of white checker squares influences the type body pattern expressed. Chiao and Hanlon (2001a) found that a checker size of ~65% of the area of the white square resulted in strong disruptive coloration (i.e. the white square was clearly expressed), while checker sizes of ~32.5% and ~97.5% elicited a weak disruptive body pattern (white square partially expressed). Checker sizes of ~13% or ~130% of the white square were said to elicit a uniform pattern, though Barbosa et al. (2004) found that checker size of about 4% to 12% of the area of the white square elicited mottled rather than a uniform body pattern. In the second experiment they found that

increasing contrast between checkers increased the expression of disruptive body patterning with the white square being most strongly expressed at 50% and 100% contrast (relative values). In their final experiment Chiao and Hanlon (2001a) found that as few as four white squares (out of the 320 black and white squares on a checkerboard) were sufficient to elicit a weak disruptive pattern with 20 white squares eliciting a strong disruptive body pattern.

The findings by Chiao and Hanlon (2001a) clearly demonstrate that cuttlefish use visual cues about the size and contrast of background features in order to produce what they perceive as the most appropriate camouflage pattern. Further the findings highlight the role that highly contrasting light coloured objects play in eliciting different body patterns. Based on this Chiao and Hanlon (2001b) went on to show that the shape or aspect ratio of white 2D objects had no effect on the expression of either uniform or disruptive body patterns, and only the size of the object effected the type of pattern produced (Chiao and Hanlon, 2001b).

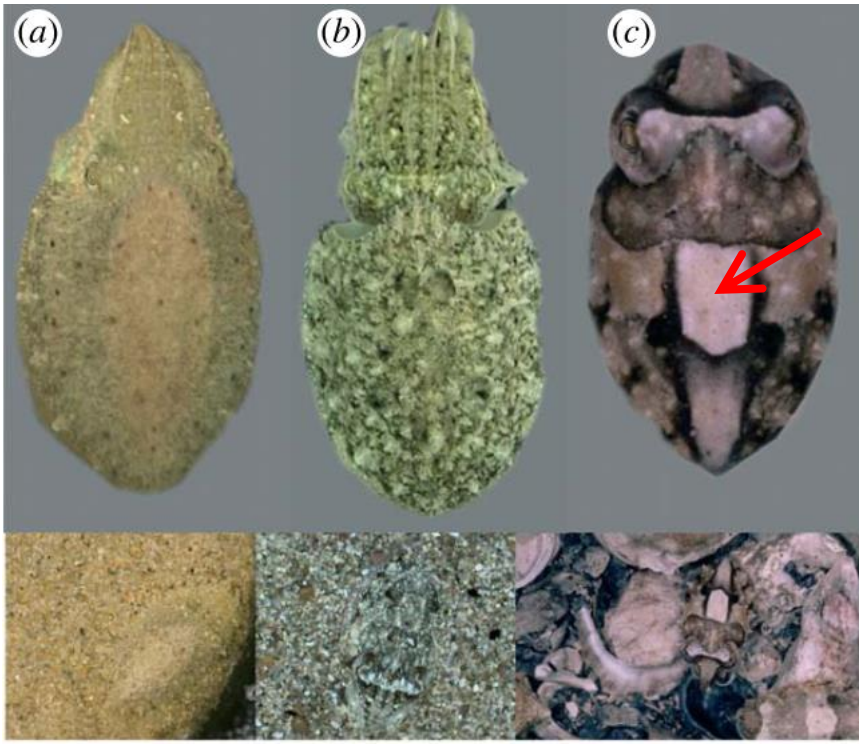


Figure 1.1: Example of (a) uniform, (b) mottle, and (c) 'disruptive' body patterns in the cuttlefish *Sepia officinalis*. The 'white square' component expressed in the disruptive pattern is indicated by the red arrow. Image taken from Hanlon et al. (2009).

Behaviourally-mediated crypsis

Behavioural background matching can take a number of different forms in different animals ranging from selecting a background that matches their own coloration and pattern, as occurs in many species of Lepidoptera (Kang et al., 2012, 2013; Kettlewell and Conn, 1977; Sargent, 1966), to actively manipulating their environment or decorating there body to increase the level of crypsis (Hultgren and Stachowicz, 2011; Wicksten, 1993). Juvenile desert tortoises (*Gopherus agassizii*) show a preference for habitats containing rocks of a similar or larger size than their own shell as this reduces predation risk through what is most likely a combination of both crypsis and masquerade (Nafus et al., 2015). Often, studies focus on behavioural choice of background at a species-level, however often this is not the case as behavioural choice can depend on the appearance of the individual. Hermit crabs for instance have been shown to be aware of their own conspicuousness on different backgrounds and alter their behaviour accordingly (Briffa and Twyman, 2011). Furthermore, when available hermit crabs will actively choose shells which match their current background (Briffa et al., 2008). Lovell et al. (2013) found that female Japanese quail (*Coturnix japonica*) choose to nest on substrate that matches the colour pattern of their eggs. Since there is a large amount of phenotypic variation in egg colour pattern it implies that individual female quail 'know' the patterning of their own eggs, thus allowing them to select the most appropriate nesting site to ensure her eggs are camouflaged (Lovell et al., 2013).

Behaviourally-mediated crypsis is also seen among colour changing animals (Ellis et al., 1997; Garcia and Sih, 2003; Kelley et al., 2012; Rodgers et al., 2010; Ryer et al., 2008). Many species of flatfish for example bury themselves in the sand to increase crypsis. For instance, Ellis et al. (1997) found that both hatchery-reared and wild sole (*Solea soles*) have an equally strong motivation to bury themselves when exposed to a sand substratum despite the fact that the reared sole had no previous experience of sand. Burial efficiency did however improve with experience (Ellis et al., 1997). Flatfish have also been shown to exhibit a behavioural preference for fine substrates over more course grained substrates (Gibson and Robb, 2000), and also choose backgrounds that match their current coloration (Ryer et al., 2008). In an experiment using English sole (*Parophrys vetulus*), northern rock sole (*Lepidopsetta polyxystra*) and pacific halibut (*Hippoglossus stenolepis*), Ryer et al. (2008) found that fish which had been placed on a light coloured sediment for 4 to 6 weeks showed a preference for light over dark coloured sediment when given a choice between the

two. In a observational field based study Tyrie et al. (2015), discovered that peacock flounder (*Bothus lunatus*) maximise crypsis by choosing backgrounds on which they can camouflage best with their limited repertoire of colour patterns. In particular they showed a preference for low contrast, neutral coloured substrates such as sand and dead coral while actively avoiding brightly coloured backgrounds such as sponges and live coral (Tyrie et al., 2015).

Purpose of this thesis

Beyond studies on cephalopods, flatfish, and chameleons, research on other animal groups capable of rapid (within seconds to minutes) colour and pattern change for camouflage is limited to a few isolated studies (e.g. Mäthger et al., 2003; Stevens et al., 2014a; Watson et al., 2014; Allen et al., 2015). Furthermore, only a handful of studies have investigated the importance of behavioural background matching in colour changing species other than cuttlefish and flatfish (e.g. Garcia and Sih, 2003; Rodgers et al., 2010; Kelley et al., 2012). This thesis therefore aims to address some of these gaps in our knowledge using the rock goby (*Gobius paganellus*) as a key organism. In particular this thesis will try to address questions regarding the ability of common intertidal fish to: a) match backgrounds that are representative of the colours found within their natural habitat, b) match a gradient of background brightness, and c) change their body pattern in response to changes in their visual background. Furthermore I will also try to determine the extent to which common intertidal species, such as the rock goby, use behavioural background matching to increase crypsis. The rock goby is a common rockpool species throughout the UK and Europe that is capable of changing its colour and lightness in less than a minute for camouflage (Stevens et al., 2014a). The habitat in which rock gobies occur is highly heterogeneous consisting of many different background types which differ in colour, lightness, and pattern. Furthermore, changes in tidal level mean they are also exposed to different groups of predators at high (e.g. fish) and low (e.g. birds) tide (Stevens et al., 2014a, 2014c). The rock goby therefore provides an ideal model organism for studying rapid colour change in species inhabiting heterogeneous habitats.

In chapter 2 I investigated the ability of rock gobies to match the colour of backgrounds that were representative of common colours found within their natural habitat, and their ability to match a range of backgrounds of different brightness. In addition to this, I also conducted choice experiments using the same backgrounds to determine if rock gobies

exhibit behavioural background matching in addition to changing colour and lightness. In chapter 3 I used the classic checkerboard background design similar to previous studies on pattern change (e.g. Ramachandran et al., 1996; Chiao and Hanlon, 2001a; Barbosa et al., 2008b) to determine if rock gobies change their body pattern in response to their visual background. Following this, I tested rock gobies on semi-natural backgrounds consisting of substrates of various sizes to determine if changing the spatial frequency (marking/object size) of background features elicited different responses in terms of pattern change. Predation pressure is a primary driver behind the evolution of different camouflage strategies and thus it is very important to consider predator perception when studying colour change (Endler, 1978; Stevens, 2007). I therefore used digital image analysis and a model of predator vision to quantify changes in colour, luminance (perceived lightness), pattern, and overall camouflage (Stevens et al., 2007; Troscianko and Stevens, 2015). In the final chapter I discuss the findings of this study in relation to past literature and suggest areas for future research.

Chapter 2: Colour change and behavioural background matching in a rockpool fish

Photo credit: Sam Smithers

Abstract

Camouflage can be achieved by both morphological and behavioural means. Many animals that use colour change to avoid detection by visually hunting predators have also been shown to exhibit some form of behavioural mediated camouflage. Colour change for camouflage maybe particularly beneficial for animals living in highly heterogeneous habitats, such as the intertidal zone, as they must cope with a diverse range of background types that differ in colour and brightness. One such species is the rock goby (*Gobius paganellus*), a common rockpool fish capable of rapidly (within one minute) changing its colour and luminance (perceived lightness) when placed on artificial backgrounds. However, until now no one has tested the ability of rockpool fish to match more natural backgrounds, nor has there been any research investigating whether rockpool fish also use behavioural background matching as a means of camouflage. In this chapter I used digital image analysis and a model of predator vision to investigate the ability of rock gobies to match the colour of sand and algae covered rock, as well as their ability to match a range of different background brightness. Moreover I also conducted choice experiments to determine if rock gobies exhibit a preference for certain backgrounds. Rock gobies rapidly changed their colour when placed on both coloured backgrounds, and also changed their luminance to match a range of grey backgrounds. However, the level of camouflage differed between backgrounds, with some background colours and brightness being easier to match than others. I also found that gobies display a strong behavioural preference for dark backgrounds over lighter ones. The results show that the ability of small rockpool fish to change colour for camouflage is not unbounded, and that they may also utilise behavioural strategies as a means of reducing risk from predators.

Introduction

Camouflage through cryptic coloration is one of the most widespread anti-predator strategies in nature (Cott, 1940; Ruxton et al., 2004; Stevens and Merilaita, 2009a; Thayer, 1909). The term crypsis is used to describe coloration that primarily prevents initial detection, and encompasses several different forms of camouflage including countershading, background matching, and disruptive coloration (Stevens and Merilaita, 2009a). By far the most common form of crypsis is background matching (Merilaita and Stevens, 2011). Background matching is when an animal's appearance matches the overall colour, lightness, and pattern of one or several background types (Stevens and Merilaita, 2009a)

Some species, such as members of the lepidoptera, have evolved under selection to match specific backgrounds (e.g. Endler, 1984; Kettlewell, 1955), while others may have compromise markings to allow camouflage on multiple backgrounds (Houston et al., 2007; Merilaita et al., 1999, 2001). For crypsis to be effective many animals exhibit behavioural background matching whereby they actively choose backgrounds that match their own body coloration and pattern (Kang et al., 2012, 2013; Kettlewell and Conn, 1977). However, although camouflage through fixed colour patterns increases survival against visually hunting predators (Bond and Kamil, 2002; Merilaita and Lind, 2005) it does carry a number of costs (Ruxton et al., 2004). For instance, fixed coloration can make thermoregulation more difficult, or prevent prey from taking advantage of opportunities available in habitats that do not match their coloration (Ruxton et al., 2004).

One way that animals may reduce these limitations is to actively change colour in response to changes in their visual background. Colour change has been documented in many animal linages including reptiles (Stuart-Fox et al., 2008), fish and amphibians (Sköld et al., 2013), crustaceans (Stevens et al., 2013, 2014c), and cephalopods (Hanlon and Messenger, 1988). Cephalopods provide perhaps the best known and well-studied examples of rapid colour change. Cuttlefish, despite being colour blind (Marshall and Messenger, 1996; Mäthger et al., 2006), are able to match the colour of different natural backgrounds (Akkaynak et al., 2013; Mäthger et al., 2008) to effectively camouflage themselves in the eyes of their predators (Chiao et al., 2011). Furthermore, cuttlefish also use visual cues, such as substrate size, contrast, and configuration, to respond to changes in background pattern by

alternating between so-called uniform, mottle and disruptive body patterns (Barbosa et al., 2008b; Chiao and Hanlon, 2001a; Chiao et al., 2007).

Colour change is particularly common amongst teleost fishes (Sköld et al., 2013), with many species being known to change colour to match the lighting conditions of their visual environment (e.g. Clarke and Schluter, 2011; Kelley et al., 2012). For instance, two sympatric species of threespine sticklebacks have been shown to change colour to better match the visual environment when moved between the pelagic and littoral zones (Clarke and Schluter, 2011). Other species change colour to match the colour and brightness of different substrates (Kelman et al., 2006; Lanzing, 1977; Ramachandran et al., 1996; Sumner, 1935). The speed of colour change does however vary considerably between species. Among flatfish for instance, species such as English sole (*Parophrys vetulus*), northern rock sole (*Lepidopsetta polyxystra*) and Pacific halibut (*Hippoglossus stenolepis*) take days to weeks to fully change colour (Ryer et al., 2008), while eyed flounder (*Bothus ocellatus*) take 2-8 seconds to match their background (Ramachandran et al., 1996).

The ability to change colour for camouflage provides a clear survival advantage (Fairchild and Howell, 2004; Sumner, 1935), however the ability of animals to match different backgrounds is not unbounded with some backgrounds being easier to match than others (Stevens et al., 2014a). As such, a number of colour changing species also exhibit some degree of behavioural background matching (e.g. Garcia and Sih, 2003; Ryer et al., 2008; Tyrie et al., 2015). The peacock flounder (*Bothus lunatus*), for example, prefers substrates that it is able to match while avoiding those it cannot (Tyrie et al., 2015).

In a recent study Stevens et al. (2014a) found that rock gobies (*Gobius paganellus*) are capable of rapid (occurring within one minute) changes in colour and luminance (perceived lightness). When placed on backgrounds of different brightness (black or white) rock gobies become darker or lighter respectively. Camouflage was better on the black background than the white one, and although the gobies showed a significant improvement in camouflage over time on the white background it may have done little to reduce the risk of predation in real terms. Moreover, when placed on a red background, rock gobies become redder in appearance, however when placed on a blue background they did not turn bluer, but rather become greyer in colour (Stevens et al., 2014a). This suggests that rock gobies may be better at matching some colours more than others, and thus their ability to camouflage

themselves on a new background will depend on the colour and brightness of the new background. The backgrounds used by Stevens et al. (2014a) were, however, artificial and there is a need for experiments using more natural backgrounds. Nevertheless, the findings of Stevens et al., (2014a) raise questions regarding whether or not fish species, such as the rock goby, also exhibit behavioural background matching to make up for their limited ability to match certain backgrounds.

With the exception of a few studies, such as Stevens et al. (2014a) and Allen et al. (2015), there is limited research on rapid colour change for camouflage in fish beyond studies on flatfish. This chapter therefore aims to address these short falls using the rock goby as a model species. The rock goby is an ideal model organism for studying rapid colour change in intertidal species, which are exposed to both marine and terrestrial predators. In the first set of experiments I investigated the ability of rock gobies to match different backgrounds which were representative of the natural colours found within rockpools, and tested if rock gobies show a behavioural preference for one colour or brightness over another. These experiments also aimed to determine whether an individual's previous background has an effect on either its ability to match a new background, or its background preference when presented with a choice between its previous background and a new one. In addition to testing change in colour, this chapter also investigated luminance change over a range of background brightness, and tested if rock gobies exhibit a behavioural preference for either dark or light backgrounds. In the choice experiments I also ask if familiarity with a background (i.e. a fish's previous background), and/or a preference for a particular background, affects the number of times an individual moves between two backgrounds. Digital image analysis and a model of predator vision were used to quantify changes in colour, luminance, and camouflage as per previously outlined methods.

Methods

The study consisted of four experiments which were carried out in-situ on Gyllyngvase beach, Falmouth, Cornwall, UK (50° 8’933.46900”N, -005° 04’907.97160”W) between December 2014 and July 2015. Fish were collected by hand and dip net from rock pools and placed in a grey bucket containing fresh seawater. All work was conducted under approval from the University of Exeter Biosciences ethics committee (application 2015/739). The field location where the experiments were conducted and fish collected is public land and no further licences or permits were needed. Following testing all individuals were measured (excluding the tail) before being returned unharmed to their original rockpool area. Rock gobies are not an endangered or protected species.

Colour and luminance change experiments

Generating the experimental backgrounds

Experiment 1

For experiment 1, two experimental backgrounds were created that were representative of the natural colours of the different substrates found in and around rockpools on Gyllyngvase beach. For this purpose photographs of the different backgrounds found along the beach were taken using a Nikon D7000 digital camera set up on a tripod (see section below on image analysis for details on camera set up). A Spectralon grey reflectance standard (Labsphere, Congleton, UK), which reflects 40% of all wavelengths between 300 and 750 nm and a ruler were included in all of the photos. Since it is not possible to print in ultraviolet (UV), photos were only taken in human visible light and not UV light. Two substrate types were chosen to generate the backgrounds; these were rock, which was often covered in green algae, and sand. These two substrates were chosen because they were distinctly different from one another in terms of overall colour. Although common in the rockpools, pebbles and small stones were not sampled because they often consisted of many different shades and colours within an area smaller than the size of rockpool fish. Wherever possible the photos were taken of rock and sand that was wet, but not submerged.

A total of 24 (12 for rock and 12 for sand) samples were selected from the photos of the natural backgrounds. The images were processed in the way described in the section

below on image analysis, though they were not mapped to avian vision, to obtain values for each of the camera colour channels (longwave (LW), mediumwave (MW) and shortwave (SW)), whereby a value of 65535 on a 16-bit scale is equal to 100% reflectance (Stevens et al., 2007; Troscianko and Stevens, 2015). These values were then converted into proportional values. Using the means of these values taken across all samples of each background type as a reference a grid of similar colours was generated for the two background types using the RGB and CYMK scales in the graphics program inkscape v0.48. The grids were then printed and photographed in human visible light. An Iwasaki eyeColour MT70D E27 6500K arc lamp which had had the UV/IR protective filter removed was used as the light source for these photos. The proportional RGB values were then calculated for each colour in the grid in the same way as before. The colours which were the closest match to the mean proportion RGB values of the natural backgrounds were then used as references to generate a second grid of colours. This process was repeated for both sand and rock until the proportional RGB values of the closest matching artificial colour was within 0.05 of the mean proportional RGB values of the natural backgrounds.

Because the aim of this experiment was to test the ability of the fish to match backgrounds that differed in hue only, it was importance to match the brightness of the two backgrounds. The green channel was used as the measure of luminance in accordance with previous work that used similar techniques to those described here (Spottiswoode and Stevens, 2011; Stevens et al., 2013). The final rock-coloured background and sand-coloured background (hereafter referred to as rock and sand) both had an approximate reflectance of 40% ± 2% in the green channel. Note that the backgrounds were designed to match the colour, but not the lightness, of the natural backgrounds. A grey scaled starting background which was the same brightness as the experimental backgrounds was created using the same method as described below.

Experiment 2

Four backgrounds of different brightness (black, dark grey, light grey and white) were generated for experiment 2. The backgrounds were generated in a similar way as those used in experiment 1. A grid of grey squares starting with RGB values of 0:0:0 (black) and increasing in increments of 2 all the way to a RGB value of 255:255:255 (white) was printed and photographed. A black and white reflectance standard with a scale bar (see section on image analysis) was included in all photos taken. The darkest grey (i.e. RGB of 0:0:0) was

used for the black background and the plain paper was used as the white background. The dark and light grey backgrounds had a reflectance of 25% and 75% relative to the black and white paper. The actual reflectance of the black and white backgrounds was ~8% and ~90% respectively, thus the actual reflectance of the dark and light grey backgrounds was ~28.5% and ~69.5% respectively. A 50% grey (actual reflectance was ~49%) was used for the starting background on which all fish were placed before starting the experiment. Printing for experiment 1 was done on HP LaserJet Tough paper (Hewlett Packard, Palo Alto, USA) while all other printing was done on Xerox Premium NeverTear waterproof paper with a Hewlett Packard LaserJet 500 color M551 PCL6 printer.

Experimental set up.

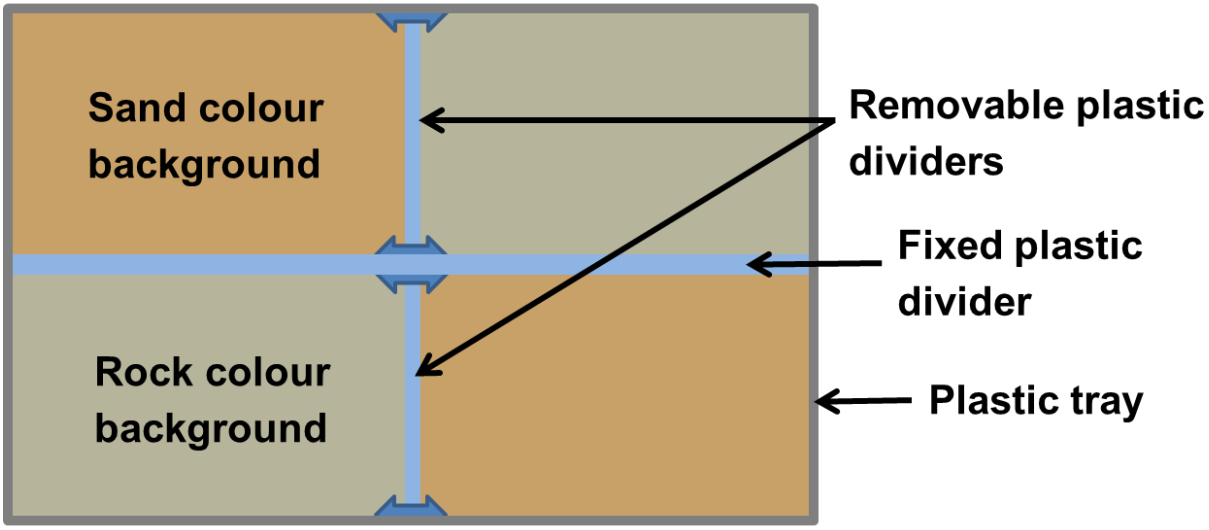
Both experiments were carried out using a 400 x 300 x 65 mm plastic tray. In experiment 1 the tray was divided lengthways into two discrete halves by a 3 mm thick acrylic wall that was fixed in place using aquarium safe silicone adhesive. The two halves were in turn divided into two sections by a removable 2 mm thick acrylic divider that was held in place by transparent slide binders that were glued to the walls using the silicone adhesive. This was done to facilitate moving the fish from one section to another without the need for further handling. This was important because previous work reported that rock gobies sometimes elicited a darkening of the skin in response to stress during handling (Stevens et al., 2014a). A similar response has also been reported in the goby *Gobius minutus* (Fries, 1942) as well as some species of crustaceans such as the fiddler crab *Uca capricornis* (Detto et al., 2008). The use of sliding doors to facilitate the movement of fish between backgrounds ensured handling was minimal thus greatly reducing any stress related colour change. Each of the four sections measured approximately 185 x 13 mm. The bottom and sides (including plastic dividers) of each section were covered by either the rock or sand background. The design and layout of the experimental tray is shown in Figure 2.1. A 360 x 250 x 50 mm white starting tray which had been covered in the grey starting background for experiment 1 was used to remove individual difference in colour between fish prior to starting the experiment.

For experiment 2 the tray was separated into four separate sections that were in turn split in half by a removable divider. Each of the eight compartments measured approximately 85 x 13 mm. The bottom and sides of the four middle compartments were covered with the

intermediate starting grey while the four outside compartments were covered with either the black, dark grey, light grey or white backgrounds as shown in Figure 2.2.

In both experiments the trays were filled with fresh seawater to a depth of approximately 20 mm and a spirit level was used to check that the trays were both level prior to starting the experiment. This was important to prevent variation in colour and brightness measurements due to water depth and to ensure that even the largest fish were fully submerged. Fresh seawater was used for each fish and the fixed acrylic walls prevented the flow of water between the different sections.

A



B

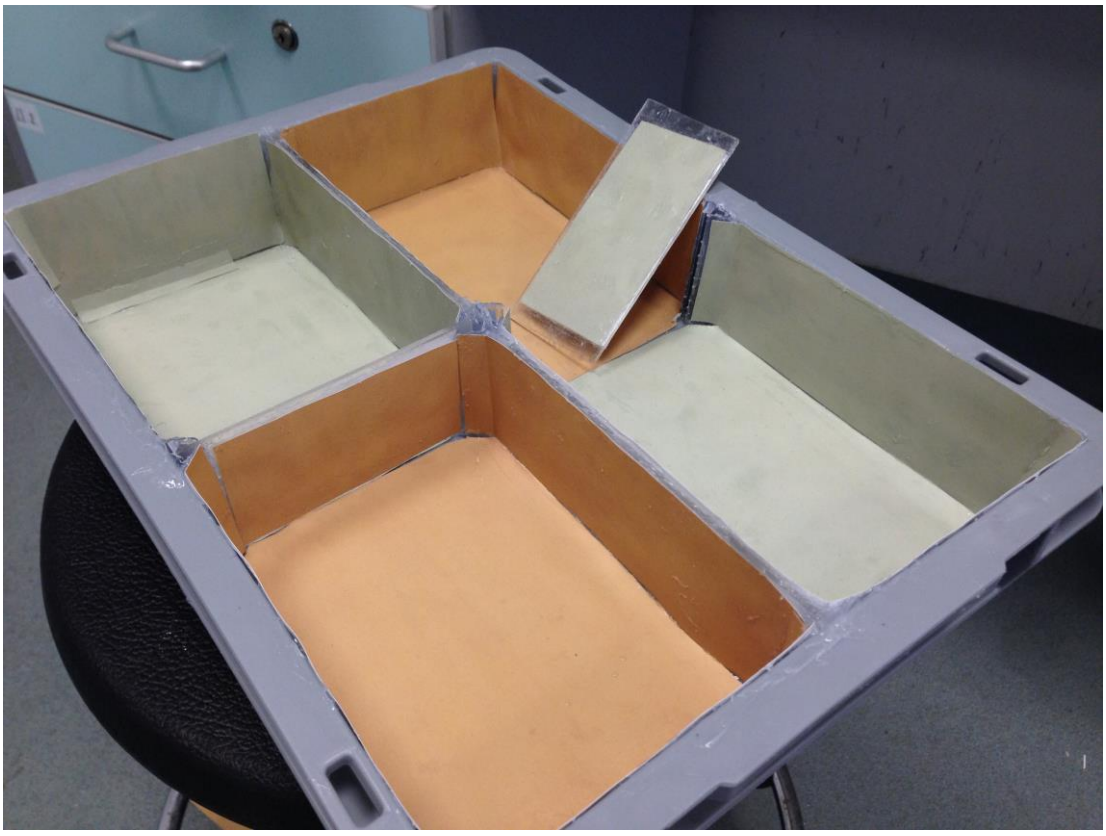
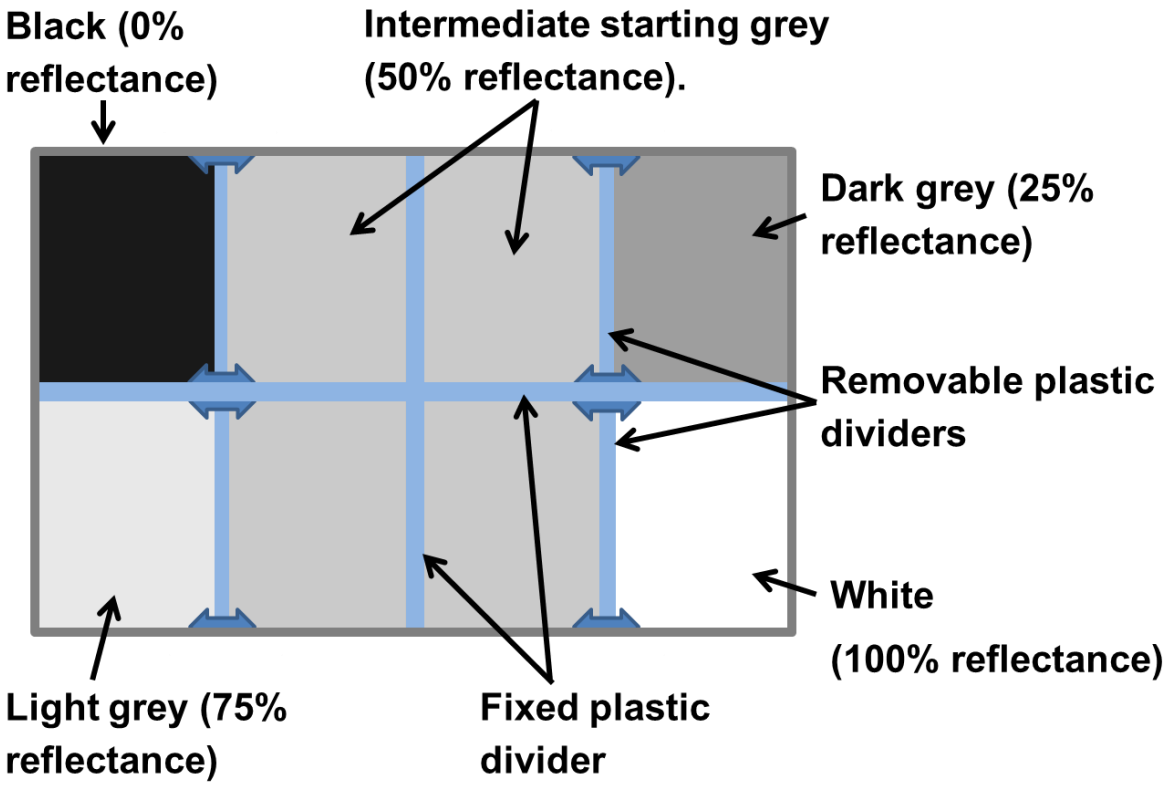


Figure 2.1: Experimental tray used for experiment 1. (A) Diagram showing the experimental backgrounds and the design of the tray used in experiment 1, and (B) final tray with one of the sliding dividers removed. Note that colours of the backgrounds may not be a true representations of the actual colours used in the experiment. Photo credit: Sam Smithers

A



B

Figure 2.2: Experimental tray used for experiment 2. (A) Diagram showing the experimental backgrounds and the design of the tray used in experiment 2, and (B) final tray, including plastic box used for housing the photographic reflectance standard, photographed during one of the trials. Note that colours of the backgrounds may not be true representations of the actual colours used in the experiment. Photo credit: Sam Smithers

Experimental procedure

A total of 20 fish were used in experiment 1 and 80 fish in experiment 2 (20 fish per background). Fish were tested in size matched blocks (to within ~20 mm) in which individuals were tested simultaneously ± 15 min. Each block consisted of two fish in experiment 1 and four fish in experiment 2.

For experiment 1, a repeated measures design was used whereby each fish was tested on both backgrounds. The first background that fish were placed on was alternated so that half were tested on rock first and half on sand first. Before starting the experiment each fish was placed on its own in the grey starting tray and allowed to acclimatise for a minimum of 15 min. This was done to remove any individual differences in colour between fish and to ensure that all of the fish acclimated to the same background before started the experiment. This was important because the fish had been collected from different rockpools, which often consisted of very different backgrounds. After the fish had been allowed to acclimatise for at least 15 min it was photographed in both visible and UV light. A net was then used to transfer the fish to the experimental tray as quickly and smoothly as possible to reduce any stress induced colour change. Stress induced colour change was easily identified as a rapid darkening of the skin during or just after handling. On the few occasions that this occurred during handling the experiment was stopped and the fish released. The experiment was then restarted with a new set of fish. Stress induced darkening was only ever observed while transferring individuals by net and never while moving them via the sliding dividers. The experiment began immediately after placing the fish on the first background. Each fish was photographed at approximately 1, 3, 5, 10, and 30 min. After being tested for 30 min on the first background each fish was photographed again (to be used as 0 min for the second background) and then moved to the next background by lifting the removable plastic divider allowing the fish to swim into the next section. The fish was gently moved by hand into the next section if it did not swim into it straight away. Fish were tested on the second background for 30 min and photographed at the same time intervals as before.

In experiment 2 each fish was only tested on one of the four backgrounds. Before starting the experiment each fish was first placed in the grey starting background and allowed to acclimatise for a minimum of 15 min before being photographed. The fish was then immediately moved across to the experimental background by lifting the divider and gently

moving the fish by hand if necessary. Fish were then photographed at 1, 5, and 30 min. Twenty fish that had been tested on either the black or the white background were used for the black versus white choice experiment (see below). Note that these fish were not measured until after taking part in the choice experiment as doing so beforehand may have stressed the fish and consequently affected the outcome of the choice test.

Background choice experiments

Experiments 3 and 4: experimental set up and procedure

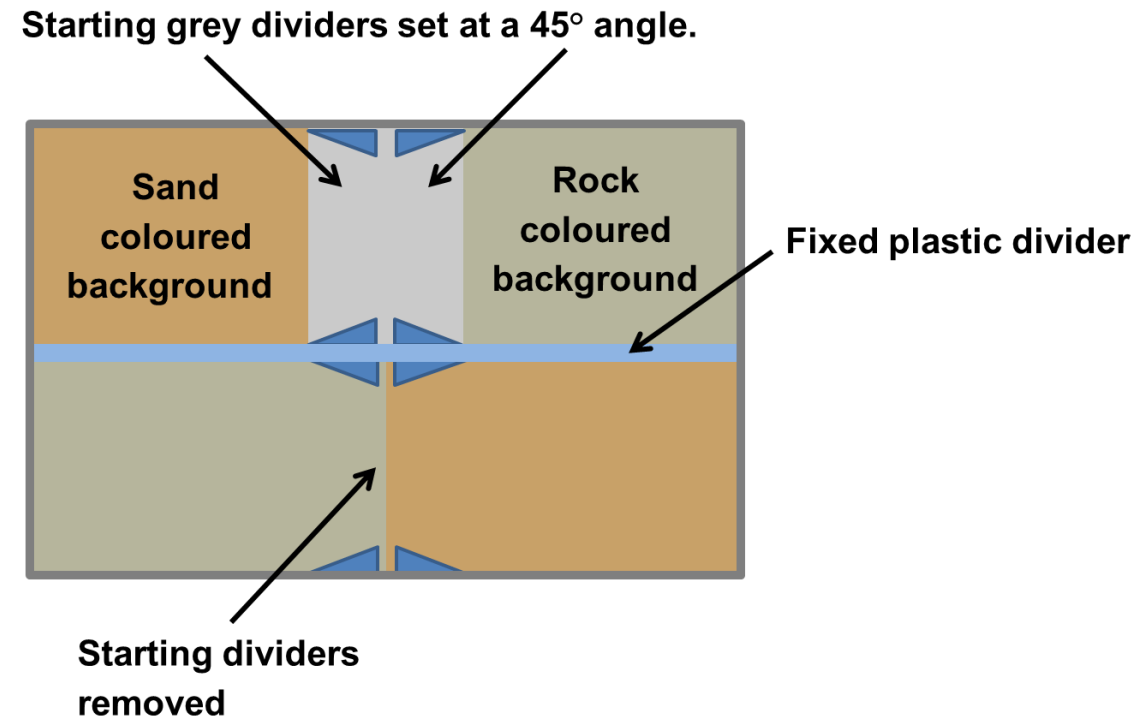
The aim of the choice tests was to see whether the fish have a preference for either sand or rock (experiment 3), or black or white (experiment 4) backgrounds and to determine whether or not the background the fish has been acclimatised to affects their preference when given a choice. A total of 40 fish were used for experiment 3 and 40 (including 20 used for experiment 2) for experiment 4 (20 per background). Prior to the experiment fish were randomly assigned to either rock or sand (experiment 3), or black or white (experiment 4) in size matched pairs and given a minimum of 30 min to acclimatise to their background and potentially change colour to match it. The acclimatisation tray was divided into eight equally sized sections which were sealed to prevent the flow of water between compartments. Note that the 20 fish that had been used in experiment 2 were not placed in the acclimatisation tray since they had already spent 30 min acclimatising to their background.

Both experiments were carried out in plastic trays which had been divided lengthways in the same way as the tray used in experiment 1. In experiment 3 half of each section was covered with the sand background and the other covered with the rock background as shown in Figure 2.3. In experiment 4 half was covered with black and the other white as shown in Figure 2.4. The two sides were separated by two sliding dividers set at a 45° angle to the bottom of the tray and a 90° angle from each other. The dividers for experiments 3 and 4 were covered with the grey starting background used in experiments 1 and 2 respectively. When in place these dividers formed a small compartment in which the fish were placed before starting the experiment. The two dividers were removed as soon as the fish had been placed between them to prevent the fish from changing its colour to match the starting grey. The trial started as soon as the grey dividers were removed. If a fish was half way between both backgrounds or made no obvious choice after removing the dividers the trial was started once the fish had completely moved onto one of the backgrounds (i.e. 100% of the fish's

body was on one colour). From this point forward a fish was said to have chosen a new background if at least 50% of its body including its head was on that background. Trials lasted for 10 min and each pair was tested almost simultaneously. To ensure that nothing was missed and the behaviour of the fish was not affected by the observer all trials were recorded using a Sony HDR-PJ810 Handycam which was positioned approximately 70 cm directly above the tray using a tripod. After being tested all fish were measured and then released. Fresh seawater was used for each fish and the tray was filled to a depth of 30 mm to ensure that the fish was fully submerged when in the starting compartment prior to starting the experiment.

Around 400 min of video footage was recorded for experiments 3 and 4. I reviewed the footage from each 10 min trial in windows media player. For each fish I recorded the first background chosen (i.e. 100% of the fish's body was on that one colour) at the start of the experiment, the amount of time spent on each background and the number of times the fish moved between the two backgrounds. A fish was said to have chosen a new background (following the first background chosen) if at least 50% of its body including its head was on that colour.

A



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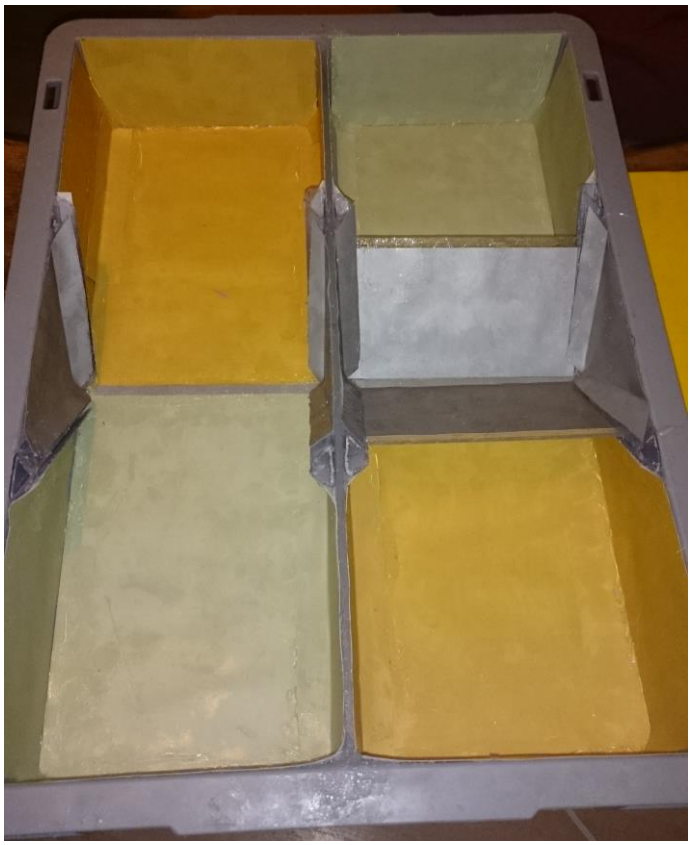
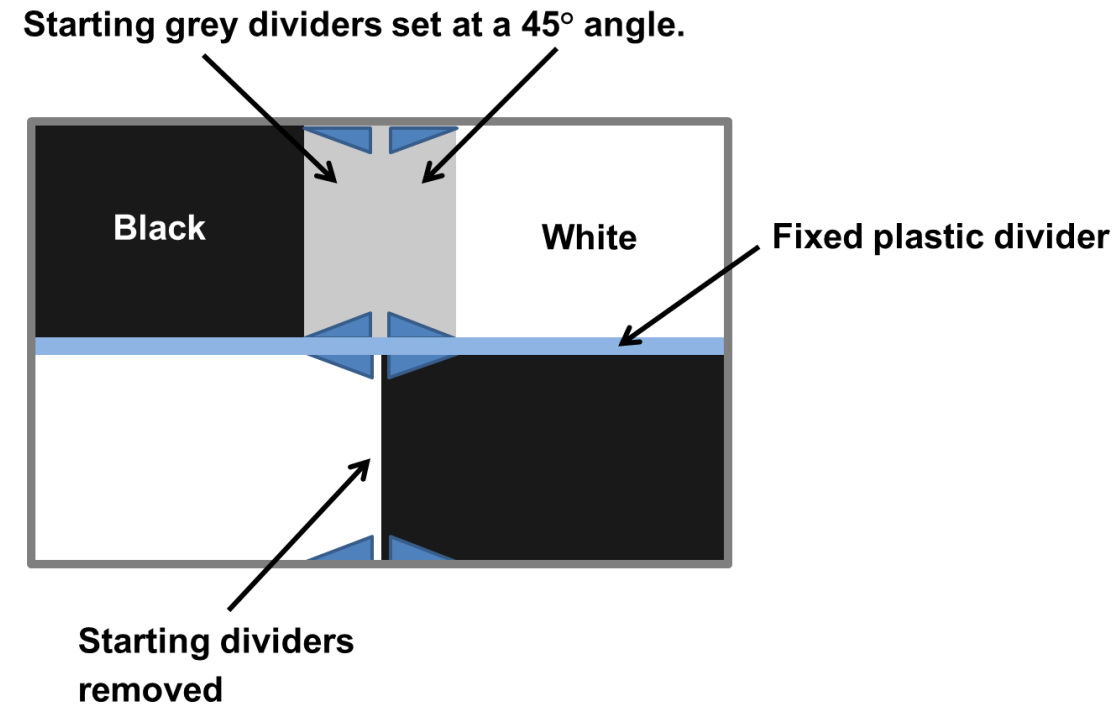


Figure 2.3: Experimental tray used for choice tests in experiment 3. (A) Diagram showing the experimental backgrounds (same as those used in experiment 1) and the design of the tray used for experiment 3, and (B) final tray with the starting dividers on one side removed. Note that colours of the backgrounds may not be true representations of the actual colours used in the experiment. Photo credit: Sam Smithers

A



B

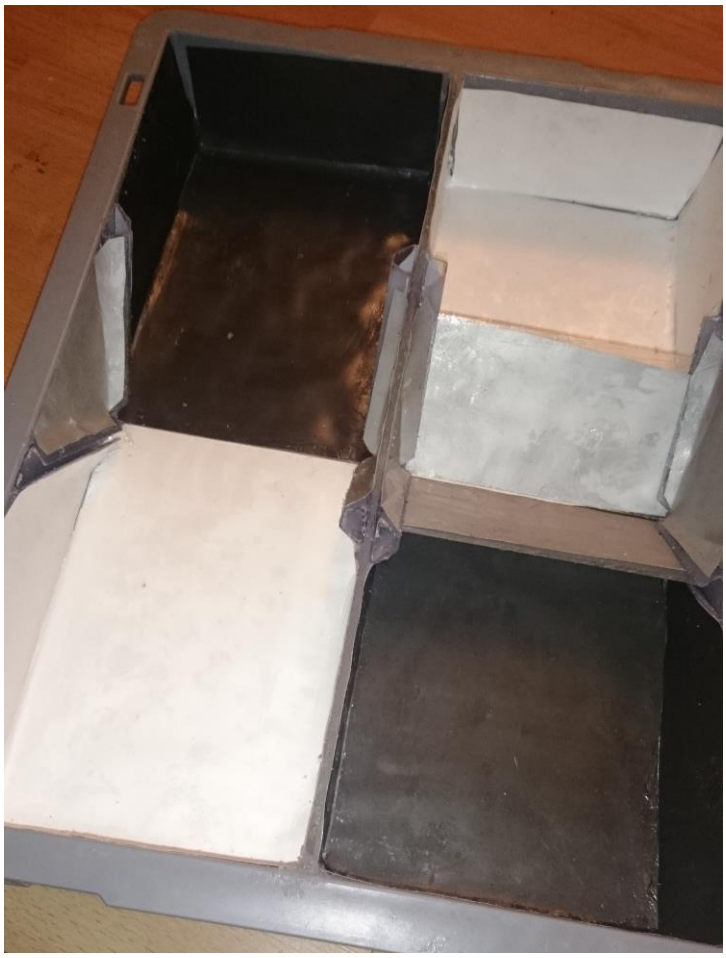


Figure 2.4: Experimental tray used for choice tests in experiment 4. (A) Diagram showing the experimental backgrounds (same as the black and white used in experiment 2) and the design of the tray used for experiment 4, and (B) final tray with the starting dividers on one side removed. Note that colours of the backgrounds may not be true representations of the actual colours used in the experiment. Photo credit: Sam Smithers

Image and video processing

All photographs were taken using a Nikon D7000 digital camera, which had undergone a quartz conversion to enable photos to be taken in both visible and UV light (Advanced Camera Services, Norfolk, UK) and fitted with a Nikon 105 mm Nikkor lens. All photos were taken in RAW format with manual white balance and fixed aperture settings using manual focus. The lens was refocused between the visible and UV photos. The human visible photos were taken using a UV/infrared (IR) blocking filter which transmits wavelengths of 400-700 nm (Baader UV/IR Cut/L Filter). The UV photos were taken using a UV pass and IR blocking filter which transmits wavelengths between 300 and 400 nm (Baader U filter). A custom made filter slider was used to quickly move between the two filters. Because overexposed photos cannot be used for image analysis, bracketing was used to ensure that at least one visible and one UV photo was correctly exposed. A black and white reflectance standard (made from 10 x 10 mm sections of zenith diffuse sintered PTFE sheet, Labsphere, Congleton, UK), calibrated to reflect 8.3% and 94.7% of all wavelengths respectively, with a scale bar was included in all photos taken (unless stated otherwise). It was important to ensure that the standard was viewed under the same light conditions as the fish. For this purpose the standard was placed in a custom made waterproof box which was positioned next to the fish in all photos. The box was made out of clear plastic which allowed both visible and UV light to pass through. A ring of aquarium safe lead inside the box was used to weigh the box down in the water next to the fish. A lid was placed over the box between photos to ensure the standard was not contaminated by dust or splashes of water. A tripod was used to position the camera directly above the fish and the standard at a height of approximately 50-70 cm. A black and silver photographic umbrella (Neewer, Guangdong, China) was used to shade the trays from direct sunlight to ensure light levels were even across the whole tray and to reduce reflectance off the water surface.

1440 RAW photos were taken in experiment 1 and 1920 photo in experiment 2 (three visible and three UV photos per time point per fish). For each time point one visible and one UV photo was chosen for image analysis using the RGB histograms provided in RawTherapee v4.1.80 (i.e. the photo with highest exposure without being over exposed). Care was taken to ensure than none of the photos chosen were over exposed. In order to perform image analysis it was first necessary to combine the visible and UV photos for each time point into a single multi spectral image using the ‘Multispectral Image Calibration and

Analysis Toolbox' developed by Troscianko and Stevens (2015). In order to correct for the non-linear responses in image values that are produced by cameras in response to changes in light levels, all of the photos were linearised with regards to light intensity (Stevens et al., 2007). Following linearisation, image values were equalised with regards to the 8.3% and 94.7% grey standards, and each of the images channels (LW, MW, SW and UV) were scaled to reflectance where a value of 65535 on a 16-bit scale is equal to 100% reflectance (Stevens et al., 2007; Troscianko and Stevens, 2015). For each image the area of the fish's body (not including the gills, eyes, or pectoral and caudal fins) was selected by hand and saved as a 'region of interest' (ROI). A 1 cm^2 sample of the background next to the fish was also selected and saved as a ROI on all images (expect those taken on the starting grey (i.e. 0 min)).

As was the case in Stevens et al. (2014a) this study analysed changes in colour and luminance as perceived by shorebirds as they are a potential predator of rockpool fish. The images were mapped to avian vision using the 'Batch Multispectral Analysis Tool' (Troscianko and Stevens, 2015), which uses spectral sensitivity data from the peafowl (*Pavo cristatus*) (Hart, 2002) under a D65 standard irradiance spectrum to convert from camera to avian colour space using a polynomial mapping technique (Stevens et al., 2007; Troscianko and Stevens, 2015). The majority of shorebirds are thought to have a 'violet' sensitive (VS) visual system (similar to the peafowl), whereby the sensitivity of the UV cone type is shifted to slightly longer wavelengths than species which have an 'ultraviolet' sensitive visual system (though violet sensitive species can still see UV light) (Ödeen et al., 2009). The peafowl is often used as a model species for modelling vision within this group. One exception to this are gulls which differ from shorebirds in that they have a UV visual system (Ödeen et al., 2009). However, the differences in the perception between these two systems is likely to be small since both the backgrounds and the fish had relatively low levels of UV reflectance. In any case, the choice of a VS system based on the peafowl is most accurate for a shorebird visual sensitivity as most gulls are not considered to be shorebirds. Compared to modelling predicted cone catch values with reflectance spectra, this mapping technique is highly accurate, with very low levels of potential error and R^2 values for each channel from 0.96 to 0.98 between derived cone catch values based on spectrometry and cameras (Pike, 2011; Stevens and Cuthill, 2006; Troscianko and Stevens, 2015).

Two variable types of 'colour' were calculated for experiment 1. First of these was saturation (the amount of a given colour compared to white light) that was defined as the distance of an object from the achromatic grey point in a tetrahedral colour space (Endler and Mielke, 2005; Stevens et al., 2014a). This was done by first standardising the values for the four colour channels to a proportion of their total in order to remove absolute variation in brightness. Next the values were converted to X, Y, and Z coordinates in a tetrahedral colour space. The more saturated a given colour is the larger the distance from the achromatic grey point at the centre of the tetrahedral (Stevens et al., 2014c). Values of saturation are on a scale of 0 to 0.75 whereby the higher the value the more saturated the colour.

Hue was used as a measure of colour type in accordance with previous studies (e.g. Stoddard and Prum, 2008; Spottiswoode and Stevens, 2011). This approach is broadly based on the way that opponent colour channels in animal vision are thought to work. Unfortunately the opponent channels that exist in birds (or indeed any animal apart from humans) are not fully known and so cannot be modelled to obtain a measure of hue. Therefore this study followed the approach set out in previous studies that used a principal component analysis to extract the main axis of variation that exists and in turn use this to determine the most logical colour channel(s) (Spottiswoode and Stevens, 2011; Stevens et al., 2014c). A PCA was performed on a covariance matrix of the standardised values for the four colour channels and the resulting principal components (PCs) were used to determine the most logical opponent model for calculating hue (conducted in IBM SPSS Statistics v21). PC1 explained 83% of the variance and was equivalent to the following colour channel: hue = $(\frac{UV+SW+MW}{3})/LW$. The lower the value of hue the redder the fish appears to avian vision. Hue and saturation were not calculated for experiment 2 because the fish were not expected to have a hue when on achromatic backgrounds, and because I was only interested in changes in luminance, and not colour type. The cone catch values for the double cone cells were used as a measure of luminance (perceived brightness) for experiment 1 and 2 as these receptors are widely believed to be involved in achromatic perception in birds (Osorio and Vorobyev, 2005). Luminance is on a scale of 0 to 1 with brighter objects resulting in higher values.

In order to determine how camouflaged each fish was against its background as perceived by birds, and how camouflage changed over time in experiment 1 a log form of the tetrachromatic version of the Vorobyev-Osorio colour discrimination model was used

(Vorobyev and Osorio, 1998). An assumption of this model is that visual discrimination is limited by receptor noise (Vorobyev and Osorio, 1998). The model uses differences in colour based on photon catch values and includes estimates of neural noise and relative photoreceptor properties. A Weber fraction value of 0.05 was used for the most abundant cone types in accordance with previous work (e.g. Stevens et al., 2014a, 2014b) and the relative proportions of the different cone types in the retina of the peafowl (LW = 0.95, MW = 1, SW = 0.86, UV = 0.45) (Hart, 2002). The model outputs 'just noticeable differences' (JNDs), whereby a value of less than 1 means that two stimuli are indiscriminable from one another, an intermediate value between 1 and 3 means that they are most likely indiscriminable but maybe discriminable under good viewing conditions, and a value greater than 3 means they will be increasingly easy to tell apart (Siddiqi et al., 2004). For experiment 2 I used an achromatic analysis based on that used by Siddiqi et al. (2004), where comparisons are based on brightness differences obtained from the double cones. When generating JNDs fish at 0 min were compared to the sample of the background at 1 min.

Statistical analysis

Statistical analysis was conducted on the data for luminance, hue, saturation, and JNDs using general linear mixed effects models in the lme4 package in R (Bates et al., 2014). Test background and time point (0, 1, 3, 5, 10, and 30 min for experiment 1, and 0, 1, 5, and 30 min for experiment 2) were included as fixed factors, and fish identification (ID) included as a random factor (fish ID was nested within test background for experiment 1 because each fish was tested on both backgrounds and may respond differently to the two colours). For experiment 1 order of testing (i.e. whether the background was the first or second the fish was placed on) was included as an additional fixed factor. I included all possible interactions in the models and used model simplification to test for significant interactions and fixed factors whereby models were fitted by maximum likelihood and compared with one another using a likelihood ratio test (LRT). For simplicity, in most cases only significant interactions are reported in the results. For experiment 1 the values for luminance, hue, and colour JNDs were log transformed while saturation was transformed using square root to ensure that the residuals were normally distributed and there was no heteroscedasticity. For experiment 2, luminance and luminance JNDs were transformed using square root. To test for differences in luminance between the fish on the different backgrounds in experiment 2 I conducted planned pair-wise comparisons using two-sample t-tests (a Welch two-sample t-test was used

when the assumption of equal variance between groups was not met) (Ruxton and Beauchamp, 2008).

For experiment 3 and 4 background preference was assessed as follows. Sign tests were used to assess if either treatment showed a significant preference for a particular background (e.g. did fish acclimated to black spend more time on black when given the choice). A Fisher's exact test was used to determine whether the acclimation background had a statistically significant effect on background preference, or lack of, as indicated by the sign test (i.e. does background preference differ between the two treatments). An exact test was used because the sample size was too small to use a chi square test or G-test (as both of these are approximate tests that require large sample sizes). Two criteria of background preference were used. These were: a) which background did the fish spend most (i.e. more than half) of their time on during the 10 min trial, and b) which background did the fish choose first at the start of the experiment. In addition to this I also analysed the number of times fish moved between the two backgrounds during the 10 min trial using a generalized linear model fitted with a quasipoisson error structure. It is worth noting that the model was originally fitted with a poisson error structure but was overdispersed hence why a quasipoisson distribution was used. All statistical analysis and graphical modelling was carried out in R (R Core Team, 2014).

Results

Colour and luminance change experiments

Experiment 1

Luminance

There was no significant effect of background (likelihood ratio test: $chisq_1$=2.16, p=0.142; Figure 2.5a), meaning that the fish were the same brightness on both backgrounds as would be expected given that the test backgrounds were matched for brightness. There was however a significant effect of time ($chisq_5$=31.71, p<0.001), and a weak significant effect of order ($chisq_1$=4.08, p=0.0434). This effect is due to an increase in luminance at 1 min for the fish tested on the rock background first, perhaps indicating that the starting grey was slightly darker than the experimental backgrounds. Since Stevens et al. (2014a) demonstrated that rock gobies are able to change colour independent of luminance this increase is most likely the result of the starting grey not being perfectly matched to the brightness of the two experimental backgrounds.

Hue

There was no significant interaction between background and order ($chisq_1$=2.51, p=0.114; Figure 2.5b) meaning that the effect of background was the same regardless of whether it was the first or second background the fish was placed on. There was, however, a significant interaction between background and time ($chisq_5$=68.73, p<0.001), whereby fish on sand became redder than those on rock (i.e. they had a lower value for hue). There was also a significant interaction between time and order ($chisq_5$=50.77, p<0.001), potentially resulting from the fact that fish on their second background had a different starting hue at 0 min to those on their first background. To test this I reanalysed the data but excluding all data points from 0 min. When 0 min was excluded from analysis there was no effect of time ($chisq_4$=1.95, p=0.746) or order ($chisq_1$=1.55, p=0.214), but there was a highly significant effect of background ($chisq_1$=30.44, p<0.001). This demonstrates that fish on sand were significantly redder than those on rock with the majority of the change in hue occurring within 1 min of being place on the background (because time is significant when 0 min is included in the model but not when 0 min is excluded). Furthermore this shows that a fish's previous background has no effect on their hue on a new background.

Saturation

There was no significant interaction between background and order ($chisq_1$=2.47, p=0.116; Figure 2.5c) meaning that the effect of background was the same regardless of whether it was the first or second background the fish was placed on. There was a significant interaction between background and time ($chisq_5$=50.21, p<0.001), whereby fish on both backgrounds became more saturated with fish on the sand background having a higher saturation than those on rock. There was also a significant interaction between time and order ($chisq_5$=51.14, p<0.001) because fish on their second background had a different starting saturation at 0 min to those on their first background. When 0 min was excluded from analysis there was no effect of time ($chisq_4$=1.13, p=0.89) or order ($chisq_1$=1.49, p=0.222), but there was a highly significant effect of background ($chisq_1$=20.14, p<0.001). Fish on sand therefore had a significantly higher saturation than those on rock with the majority of the change in saturation occurring within the first minute of being place on the background. Furthermore this shows that a fish's previous background has no effect on their saturation on a new one.

Colour JNDs

For JNDs there was a significant interaction between background, time, and order ($chisq_5$=14.2, p=0.014; Figure 2.5d). In order to better understand this interaction I chose to analyse the JNDs between the fish and their first background (rock first and sand first) and their second background (rock second and sand second) separately. Using this new approach there was a significant interaction between background and time for the fish on their first background ($chisq_5$=22.91, p<0.001) but not for their second background ($chisq_5$=5.18, p=0.394). This can be attributed to the fact that JNDs decreased for fish on sand but increased or stayed the same for fish on rock when they were the first backgrounds the fish were tested on (i.e. the effect of time on JNDs depended on background). Conversely JNDs decreased with time for fish on both rock and sand when they were the second backgrounds they were tested on (i.e. the effect of time was the same for fish on both backgrounds). Background and time both had a significant effect on JNDs for fish on their second background ($chisq_1$=18.24, p<0.001 and $chisq_5$=42.78, p<0.001 respectively). To test whether a fish's previous background has an effect on their camouflage on a new background I analysed the data for rock (rock first and rock second) and sand (sand first and sand second) separately. There was a significant interaction between order and time for fish tested on the rock background ($chisq_5$=19.96, p=0.001). This interaction is the result of JNDs increasing and/or staying the

same when fish were tested on rock first but decreasing when fish were tested on rock second (i.e. the effect of time was different depending on the order fish were tested on rock). When fish were placed on the sand background there was no interaction between order and time ($chisq_5$=1.62, p=0.898), nor was there any overall effect of order ($chisq_1$=0.002, p=0.967). There was a significant effect of time ($chisq_5$=41.8, p<0.001). This demonstrates that a fish's previous background has no effect on its ability to camouflage on a new one. Any effect of order in the overall model can therefore be attributed to the high JNDs at 1 min for the fish tested on rock second. These high JNDs are because the fish were adapted to camouflage themselves to the sand background at time 0 (as they were tested on sand first) and not the rock background. Within one minute their JNDs had fallen to within the same range as those displayed by the fish tested on rock first.

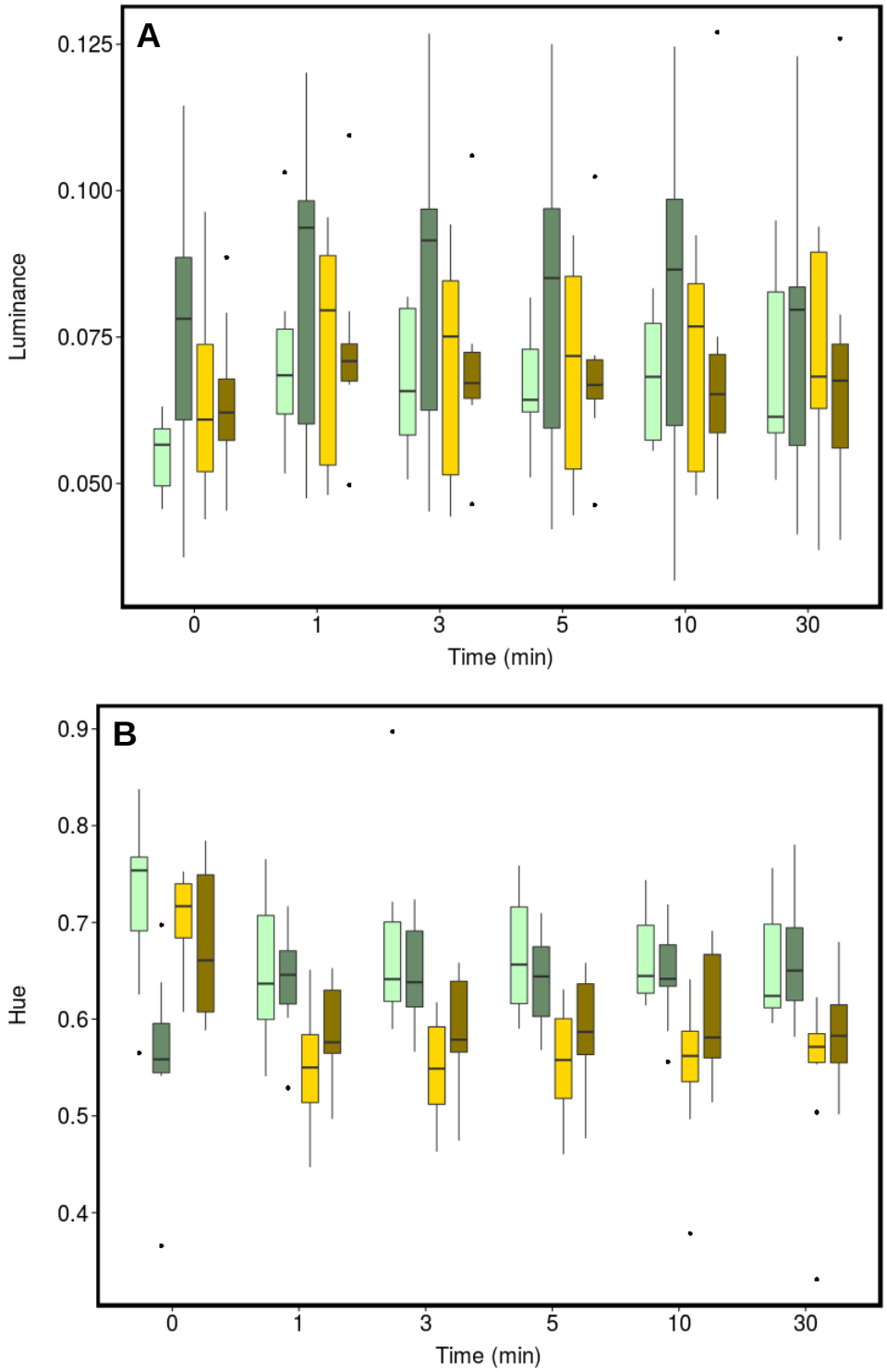


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Figure 2.5 continued…

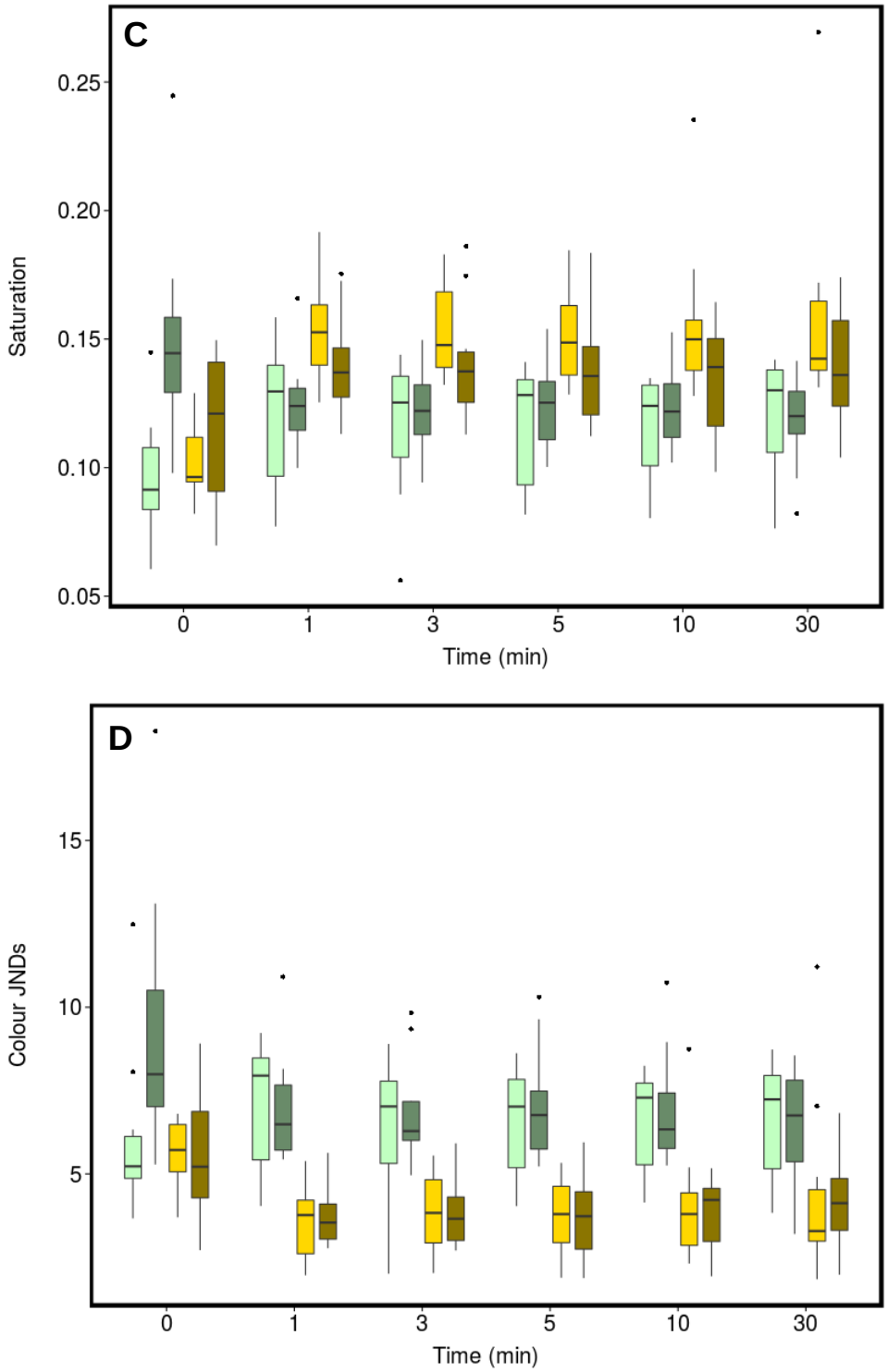

Figure 2.5: Changes in (A) luminance, (B) hue, (C) saturation, and (D) colour JNDs (based on Vorobyev and Osorio (1998)) for rock gobies placed on the 'sand' or 'rock' coloured backgrounds in experiment 1 at the start (0 minutes) and 1, 3, 5, 10 and 30 minutes. There was no difference in luminance between fish, but fish on 'sand' were redder and more saturated than those on 'rock'. Fish were more camouflage on 'sand' than on 'rock'. Graphs show medians plus inter-quartile range (IQR), whiskers are lowest and highest values that are within 1.5*IQR from the upper and lower quartiles, outliers are shown by dots. The colour of the box indicates the colour of the test background and whether it was the first or second background the fish were placed on: = rock first, = rock second, = sand first, and = sand second.

Experiment 2

Luminance

For luminance there was a highly significant interaction between background and time ($chisq_9$=193.62, $p<0.001$; Figure 2.6a), whereby fish tested on the light grey and white backgrounds increased their luminance after the first minute while fish tested on the black background decreased their luminance. There was no change in the luminance of fish tested on the dark grey background. To test for differences in luminance between the fish on the different backgrounds I performed planned pair-wise comparisons using two-sample t-tests, the results of which are presented in Table 2.1. There was no difference in luminance between fish at 0 min, with the exception of light grey versus white which were found to be statistically significant at 0 min. However, I suspect that this difference was due to an issue with the experimental tray and was not due to a difference in the brightness of the starting greys. This is because the sliding divider separating the starting grey and the white background was not a perfect fit and left a small gab at the bottom through which some of the smallest fish may have been able to see the white background during the acclimatisation period, thus why some of the fish were slightly paler at the start than they otherwise would have been. Importantly, this differences does not change the interpretation of the overall results since rock gobies have already been shown to change luminance within 1 min of being placed on a white background (Stevens et al., 2014a). At 1, 5, and 30 min there was a significant difference in luminance between the fish on the black and dark grey, the dark grey and light grey, and the light grey and white backgrounds.

Table 2.1: Results from the two-sample t-tests used for the planned pair-wise comparisons to test for differences in luminance between rock gobies placed on the black, dark grey, light grey, and white backgrounds in experiment 2. A Welch two-sample t-test was used when the assumption of equal variance between groups was not met. * = result is statistically significant (i.e. $p < 0.05$). (ns) = result is not statistically.

Planned pair-wise comparisons	**Time Point**			
	0 min	**1 min**	**5 min**	**30 min**
Black vs. dark grey	T= 0.08 df= 38 p= 0.935 (ns)	T= -6.89 df= 38 p<0.001*	T= -7.3 df= 38 p<0.001*	T= -5.45 df= 38 p<0.001*
Dark grey vs. light grey	T= -0.76 df= 38 p= 0.455 (ns)	T= -5.5 df= 38 p<0.001*	T= -4.05 df= 31.839 p<0.001*	T= -4.82 df= 27.31 p<0.001*
Light grey vs. white	T= -2.18 df= 38 p= 0.036*	T= -3.08 df= 38 p= 0.004*	T= -2.74 df= 38 p=0.009*	T= -2.58 df= 38 p=0.014*

Luminance JNDs

For JNDs there was a significant interaction between background and time ($chisq_9$=18.81, p=0.027; Figure 2.6b). In order to see the effect of each background on camouflage over time I analysed the change in JNDs over time for each background separately. On the white background there was a significant decrease in JNDs over time ($chisq_3$=19.49, p<0.001), but not when the data points for time 0 were removed ($chisq_2$=2.36, p=0.308) showing that the majority of change in JNDs occurred within the first minute. On the light grey background there was also a significant decrease in JNDs over time ($chisq_3$=11.94, p=0.008), but not when 0 min was excluded from the model ($chisq_2$=4.02, p=0.134), again showing that JNDs decreased between 0 and 1 min before levelling off. There was no change in JNDs over time for fish on the dark grey background ($chisq_3$=1.13, p=0.771), but there was a significant decrease in JNDs over time for fish on the black background ($chisq_3$=14.42, p=0.002; compared to $chisq_2$=0.37, p=0.83 when 0 min was excluded from analysis).

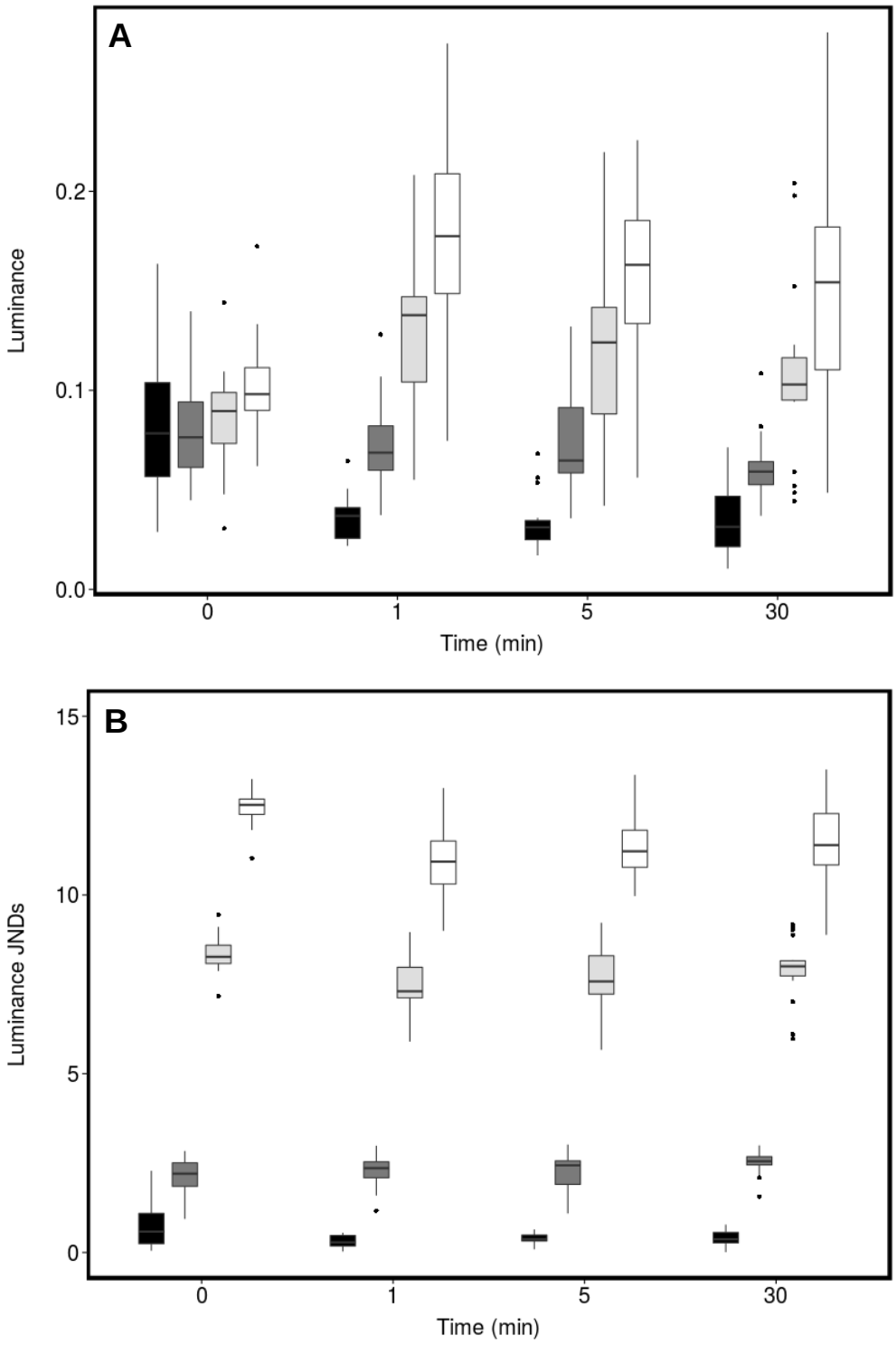

Figure 2.6: Changes in (A) luminance, and (B) luminance JNDs (based on Siddiqi et al. (2004)) for rock gobies placed on the black, dark grey, light grey, and white backgrounds in experiment 2 at the start (0 minutes) and 1, 5, and 30 minutes. Rock gobies became lighter when placed on the white and light grey backgrounds and darker on the black background. Fish placed on the white, light grey, and black backgrounds improved their level of camouflage within 1 minute. Fish on dark grey did not change their luminance, nor did they become more camouflaged. Graphs show medians plus inter-quartile range (IQR), whiskers are lowest and highest values that are within 1.5*IQR from the upper and lower quartiles, outliers are shown by dots. The colour of the box indicates which background the fish were placed on: = black, = dark grey, = light grey, and = white.

Background choice experiments

Experiment 3

Total time spent on each background

From figure 2.7a the fish acclimatised to the sand background (sand fish) appear to have a weak preference for the sand coloured background and spent on average ~65% of their time on that colour. This weak preference is somewhat supported by the fact that significantly more of the sand fish spent more than half of their time on the sand background (Sign test: p=0.041, n= 20). In comparison, fish which had been acclimatised to the rock background (rock fish) displayed no overall preference for either background (Sign test: p=0.824, n= 20) spending on average ~56% of their time on sand. However, although this suggests that acclimatisation has an effect on background preference this is not statistically significant (Fisher's Exact Test: OR= 2.4, p= 0.32, n= 40). This means overall background preference was not found to depend on the background the fish had been acclimatised thus the apparent preference of the sand fish for the sand background should therefore be interpreted with extreme caution.

First background chosen

Table 2.2 shows the number of individuals which chose either the sand or the rock background as their first background at the start of the time trial. Neither the sand nor the rock fish showed any preference for choosing either background first (Sign test: p= 0.824, n= 20, and p=1, n= 20 for sand and rock fish respectively). It is therefore not surprising that acclimation background had no effect on background preference (Fisher's Exact Test: OR= 1.22, p= 1, n= 40).

Number of background switches

Acclimatisation to either background had no effect on the number of times fish moved between the black and white backgrounds during the 10 min trial (F-test: $F_{1,38}$= 0.05, p= 0.823; Figure 2.7b).

Table 2.2: Number of times 'rock' and 'sand' were the first background chosen by rock gobies that had been acclimatised to either the rock (rock fish) or the sand (sand fish) coloured background.

Acclimatisation background	No. of times background was chosen first	
	Rock	**Sand**
Rock fish	10	10
Sand fish	9	11

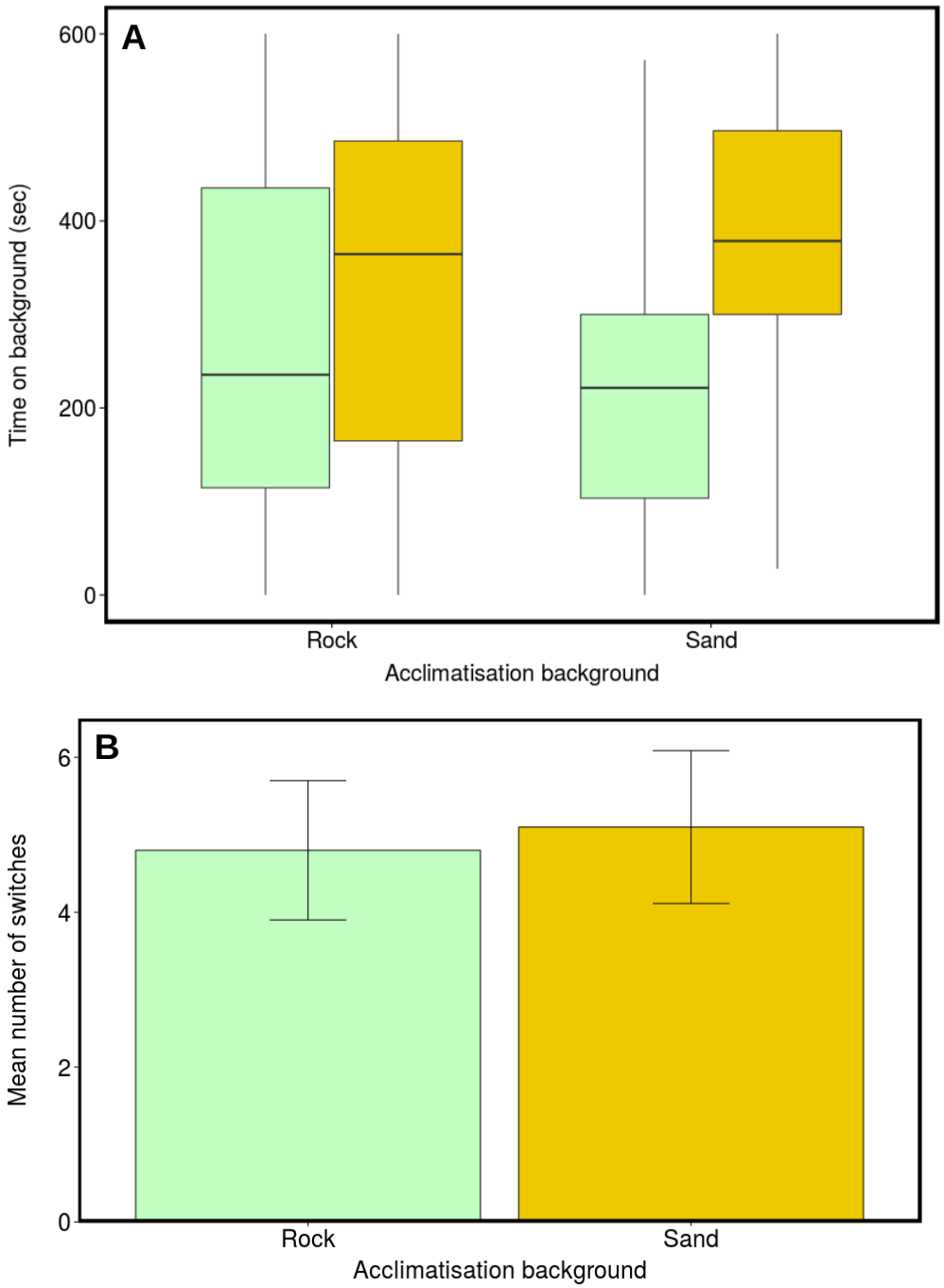

Figure 2.7: Plots showing (A) the amount of time rock gobies spent on 'rock' and 'sand', and (B) the number of times fish switched background during the 10 minute trial, for fish acclimatised to either the 'rock' or 'sand' coloured background. Acclimatisation background did not have a significant effect on background preference nor did it affect the number of times fish moved between the two backgrounds. Graph A show medians plus inter-quartile range (IQR), whiskers are lowest and highest values that are within 1.5*IQR from the upper and lower quartiles, outliers are shown by dots. Graph B shows means plus standard errors.
In panel A: = time spent on rock, and = time spent on sand.

Experiment 4

Time spent on each background

When given the choice all fish had a very strong overall preference for the black background (Sign test: $p= 0.01182$, $n= 20$, and $p<0.001$, $n= 20$ for black and white acclimatised fish respectively). Moreover, this preference was the same regardless of which background the fish had been acclimatised to (Fisher's Exact Test: OR= 0.34, $p= 0.342$, $n= 40$; figure 2.8a). Black fish spent on average ~84% of their time on black while white fish spent on average ~79% of their time on the black background.

First background chosen

Table 2.3 shows the number of individuals which chose either the black or the white background as their first background at the start of the experiment. In general, more fish appear to have chosen the black background first. This preference was statistically significant for fish acclimated to the black background (Sign test: $p<0.001$, $n= 20$) but not for those acclimated to the white background (Sign test: $p= 0.1153$, $n= 20$). However, despite this apparent difference, the effect of acclimatisation background was not statistically significant (Fisher's Exact Test: OR= 3.73, $p= 0.235$, $n= 40$). Both groups can therefore be said to be more likely to choose black as their first background, but this preference was strongest in fish which had been acclimated to black.

Number of background switches

As was the case in experiment 3, acclimatisation to either background had no effect on the number of times fish moved between the black and white backgrounds during the 10 min trial (F-test: $F_{1,38}= 0.0706$, $p= 0.7919$; Figure 2.8b).

Table 2.3: Number of times black and white were the first background chosen by rock gobies that had been acclimatised to either the black (black fish) or the white (white fish) background.

Acclimatisation background	**No. of times background was chosen first**	
	Black	**White**
Black fish	18	2
White fish	14	6

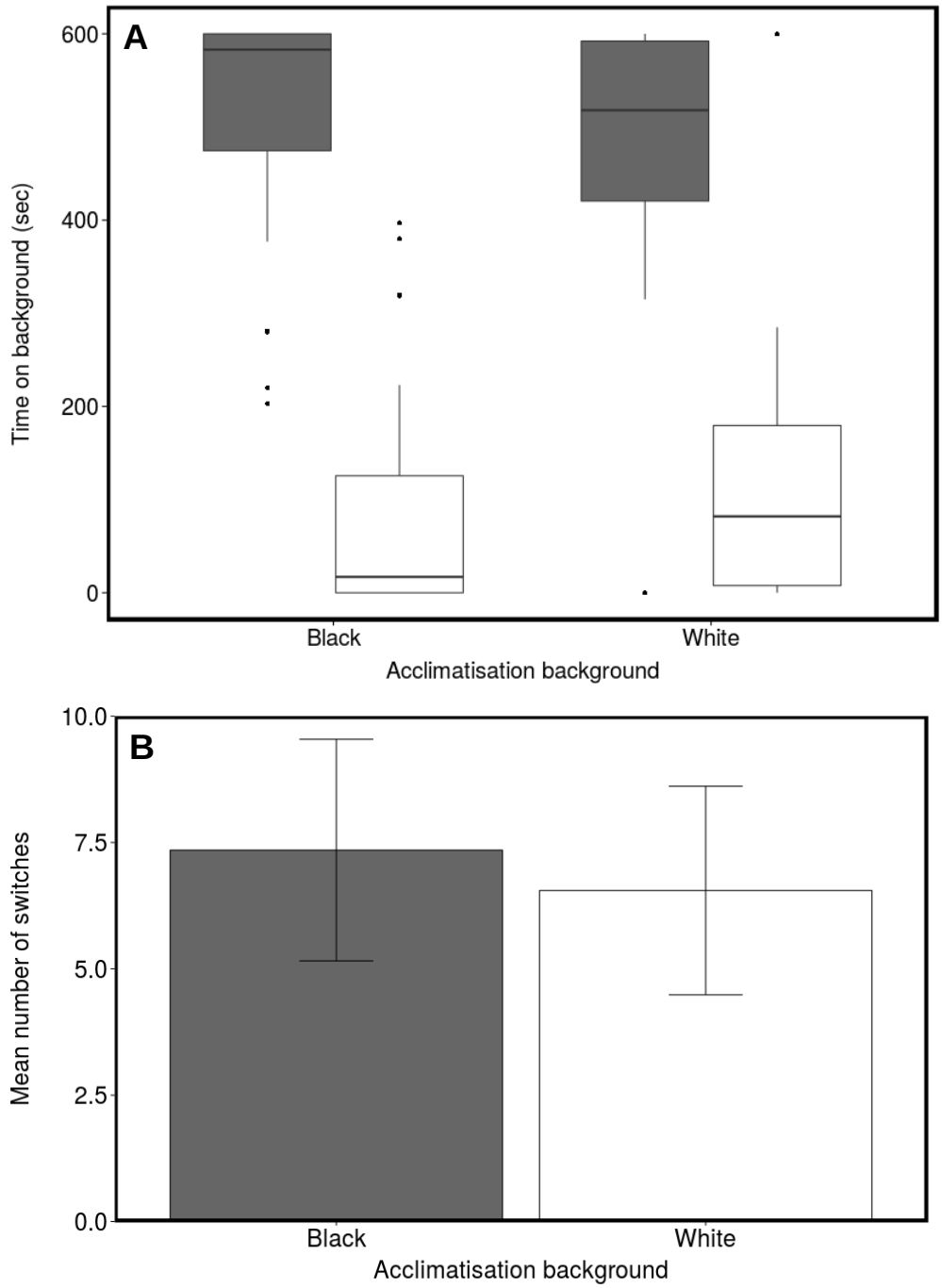

Figure 2.8: Plots showing (A) the amount of time rock gobies spent on black and white, and (B) the number of times fish switched background during the 10 minute trial, for fish acclimatised to either the black or the white background. The majority of rock gobies spent more of their time on the black background irrespective of which background they were previously placed on. Acclimatisation background did not affect the number of times fish moved between the two backgrounds. Graph A show medians plus inter-quartile range (IQR), whiskers are lowest and highest values that are within 1.5*IQR from the upper and lower quartiles, outliers are shown by dots. Graph B shows means plus standard errors.

In panel A: = time spent on black, and = time spent on white.

Discussion

In experiment 1 rock gobies rapidly changed colour on both backgrounds by becoming redder and more saturated (even on the green rock background). The amount of colour change was greatest on the sand background whereby fish were much redder and more saturated than fish on the rock background as perceived by avian predators. This result is not unexpected given that it has already been shown that rock gobies are able to turn redder in response to their background (Stevens et al., 2014a), through the fact that the fish also become redder on the rock background was more surprising. Perhaps this was because both backgrounds were perceived by the fish as being redder than the grey starting background. The luminance of the fish was the same on both backgrounds supporting the results of Stevens et al. (2014a) that rock gobies are able to change colour whilst keeping their luminance the same.

In terms of overall camouflage, as perceived by birds, the fish on their first background become more camouflaged on sand after 1 min, but camouflage stayed the same or decreased on the rock background. Fish improved there camouflage within 1 min of being moved either from sand to rock, or from rock to sand, and final camouflage was not affected by order of testing. Therefore an individual's previous background had no effect on its ability to change colour to match a new background nor did it affect overall camouflage on the new background. Moreover, the speed of change is important because it may allow gobies to move across multiple background types while minimising the amount of time that the fish is contrasting against its background. It can thus be hypothesised that rapid colour change helps to reduce predation risk in heterogeneous habitats.

During the course of the experiment fish were exposed to three different backgrounds (starting grey followed by rock and sand) all of which differed greatly in colour. Flatfish and other teleosts are known to change colour more rapidly following repeated background changes (Burton and O'Driscoll, 1992; Burton, 2010; Healey, 1999; Sumner, 1911). For instance, repeated background reversals have been shown to increase melanophore response rates in flatfish (Burton and O'Driscoll, 1992; Burton, 2010). There was no evidence to suggest that repeated background changes had any effect on the speed of colour change in this experiment although many more background changes would most likely be needed in order to result in any meaningful increase in the speed of colour change. Furthermore the methods used in this study would be unable to detect any change in speed faster than 1 min.

However, neither this study nor (Stevens et al., 2014a) looked at long term colour change which may occur over many hours or even days and it is possible that repeated background exposure could increase the speed of long term change. However, compared to rapid changes in colour, the protective value provided by long term changes in the level of camouflage would be predicted to be much lower, or even none existent, given that rock gobies have the potential to encounter many different backgrounds in a very short period of time. The repeated measures design in experiment 1 also shows that rock gobies can not only change colour rapidly, but that they can reverse this change in colour just as quickly. For instance, fish tested on sand first became redder between 0 and 1 min, but then become less red (and matched the hue of the fish tested on rock first) after being moved to the rock background thus demonstrating that this change in colour is fully reversible in the same amount of time.

Overall, fish were better at matching the more natural colours used in experiment 1 than they were the artificial colours used in Stevens et al. (2014a). It is interesting that rock gobies were more camouflaged on the sand coloured background than on the green rock coloured background, which supports the idea that some colours may be easier to match than others. There may be numerous reasons for this but one hypothesis is that there is likely to be a greater selection pressure for fish to match sand like colours compared to the colours of rock and green algae. This is due in part to the nature of the habitats in which these colours are most predominant. In my study site the greenish grey colour of the rock background is most associated with the rocks and green algae found within rockpools. Since rockpools are extremely heterogeneous in their substrate composition there is a range of different colours and so selection pressure to match any single colour is likely to be minimal. Alternatively, although not necessarily mutually exclusive, local adaptation may also influence an individual's background matching ability. However, since it is not known whether rock gobies migrate between different areas during either their juvenile or adult stage, it is not possible to reliably speculate about local adaptations without also sampling fish from different coastal areas. This does however raise intriguing questions for future research.

In heterogeneous habitats such as rockpools body pattern may be more important than overall colour (see chapter 3). Moreover rockpools provide fish with numerous places to hide from predators such as under stones or within rock crevices. In comparison sand was one of the single most common substrates on Gyllyngvase beach and is found on both rocky and sandy shores. Habitats comprising mostly of sand tend to be homogeneous and have few

places for animals to take shelter. A number of flatfish species are known to exhibit a burial response when on sand to increase crypsis (Ellis et al., 1997; Fairchild and Howell, 2004; Ryer et al., 2008) but no such behaviour has ever been documented in rock gobies. Furthermore selection pressure by predators in heterogeneous environments such as rockpools would be lower than in homogeneous habitats dominated by sand since prey detection has been shown to be more difficult in complex habitats (Bond and Kamil, 2006; Dimitrova and Merilaita, 2012; Stoner and Titgen, 2003). It is therefore plausible that there would be a higher predation risk from birds on sand than in rocky shores thus there would be a higher selection pressure to match the colour of sand than green algae covered rock. This is made more important by the fact that the action of waves and currents may force rock gobies on to areas of the shore, such as sand, where they may otherwise not choose to go. Furthermore, the ability to become redder may also allow gobies to camouflage themselves against a range of other substrate types such as red or brown coloured rock, as well as red algae, both of which are common in rockpools.

In experiment 2, rock gobies placed on white or light grey became lighter in colour, while individuals placed on black became darker. There was no change in luminance observed for fish on the dark grey, suggesting that perhaps the fish were already dark enough to match the dark grey in the first place. Indeed, even before being exposed to the backgrounds camouflage was much better on the darker backgrounds than on the lighter ones. Camouflage was found to improve after 1 min for fish on the white, light grey and black backgrounds but did not change for fish on the dark grey. The improvement in camouflage on the black background differs from previous research on rock gobies which reported no significant change in camouflage on black (Stevens et al., 2014a). Nevertheless, overall the results confirm the findings of Stevens et al. (2014a) that rock gobies are better at matching the brightness of dark backgrounds than lighter ones.

One possible explanation for this relates to the mechanism behind colour change in fish. Rapid physiological colour change is mediated by the movement of pigment organelles within chromatophores (specialised pigment cells) (Sköld et al., 2013). The luminance of the skin is controlled by melanophores which contain the pigment melanin within organelles called melanosomes (Burton, 2002; Sköld et al., 2013). By dispersing the melanosomes throughout the melanophores the fish is able to become darker in appearance and thus match the dark backgrounds. The degree of darkening is mostly limited by the amount of melanin

present within each cell. However, when the dark melanosomes are aggregated the melanophores become not only paler but also more transparent (Sköld et al., 2013). This means that to a certain extent the ability to match very pale backgrounds may be limited by how transparent the fish is, thus placing a potential limitation on colour change. Interestingly, some fish, such as the two-spotted goby (*Gobiusculus flavescens*), have been found to change colour using internal chromatophores in addition to the more widely known epidermal chromatophores (Sköld et al., 2008).

It is interesting that the rock gobies were darker on the light grey than the white, despite the fact that both backgrounds were much lighter than the maximum lightness the gobies were capable of achieving, thus it would be expected that both backgrounds would elicit the same change in luminance (i.e. the greatest increase in luminance possible). It is also interesting that the rock gobies did not change luminance in response to the dark grey background suggesting that perhaps the fish did not perceive a big enough difference between the 50% reflectance starting grey and the 25% reflectance dark grey to warrant a change in luminance. This is however unlikely given that most animals are thought to perceive absolute brightness on a none-linear scale (Cronin et al., 2014). This means the difference in brightness between the 50% and 25% greys would be perceived as being greater than the difference between the 50% and the 75% greys despite the absolute difference between them being exactly the same (Cronin et al., 2014). Rock gobies are naturally dark anyway and so it is more likely that they were already dark enough to match the dark grey background without any need for additional luminance change.

Figure 2.9 shows the final JNDs between the fish and all four grey backgrounds used in experiment 2. Even when they are adapted to white gobies are more camouflaged on the black and dark grey than they are the two lighter backgrounds. What is surprising is that the fish tested on the white and light grey backgrounds are more camouflaged on the dark grey background than the individuals which were actually placed on dark grey. On all backgrounds other than black, the fish were always darker than the background they were on despite being able to increase their luminance further. The most likely cause of this is that many of the fish had dark contrasting body patterns (see chapter 3). Since the whole body was selected as the ROI during image analysis the darker patterns would have reduced the mean luminance of the fish. The implication of this is that while the overall luminance of the fish may have matched the background, the dark patterns would have meant that the fish

always appeared darker than most of the backgrounds in terms of mean luminance. Overall camouflage to the background is therefore likely to depend not only on the colour or luminance of the fish, but also on body pattern, and how these markings function and interact with the background (see chapter 3).

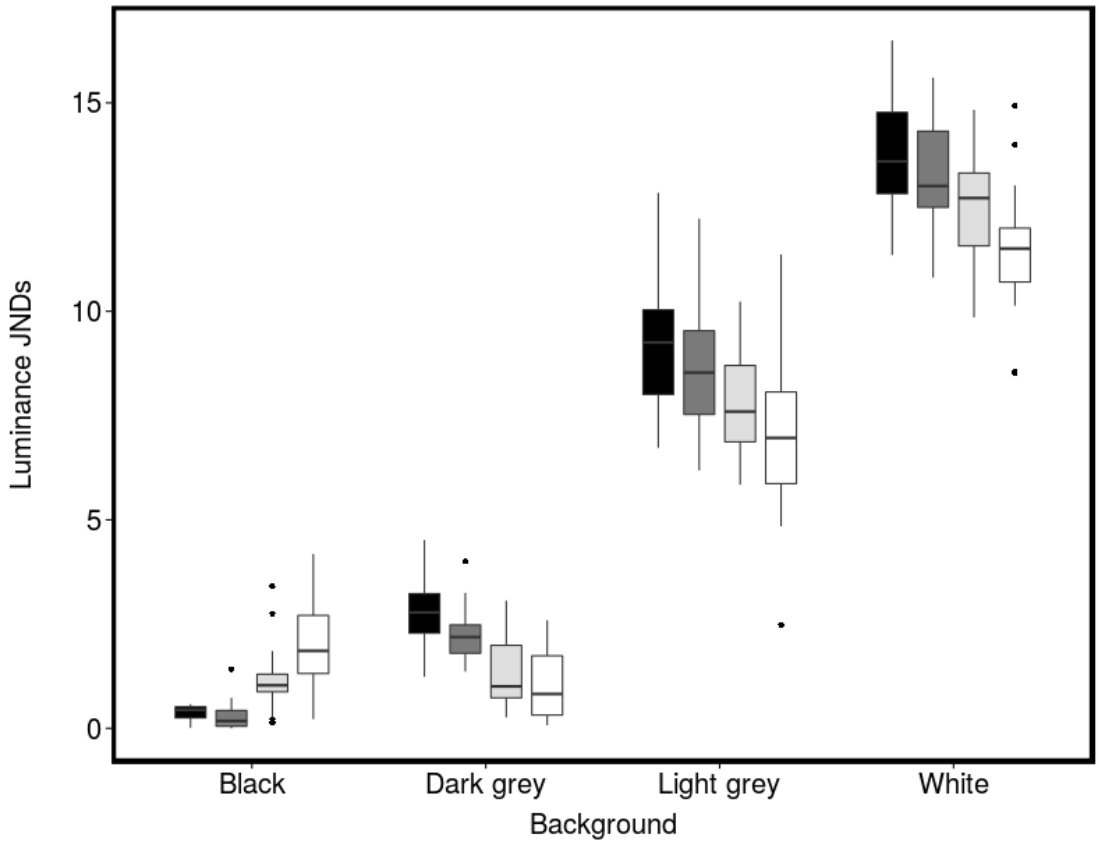

Figure 2.9: Luminance JNDs (based on Siddiqi et al. (2004)) of rock gobies placed on the black, dark grey, light grey, and white backgrounds in experiment 2, when viewed by birds against their own background and each of the other backgrounds at 30 minutes. Rock gobies are more camouflaged when viewed against the two darkest backgrounds than the lighter ones irrespective of which background they had actually been placed on during the experiment. Graph show medians plus inter-quartile range (IQR), whiskers are lowest and highest values that are within 1.5*IQR from the upper and lower quartiles, outliers are shown by dots. The colour of the box indicates which background the fish had been placed on in experiment 2: = black, = dark grey, = light grey, and = white.

Rock gobies displayed a strong preference for the dark background over the lighter one (in terms of the amount of time spent on each) regardless of which one they had been acclimated to. Furthermore, individuals acclimatised to both backgrounds tended to choose black as their first background. This indicates that an individual's previous background does not appear to influence background preference in the future. This suggests that the preference for dark backgrounds may be an innate behaviour. Overall this result is in accordance with other studies which found that other species of fish and amphibians also have a preference for dark backgrounds (Bradner and McRobert, 2001; Garcia and Sih, 2003; Kjernsmo and Merilaita, 2012). It does however differ from choice experiments performed on three species of juvenile flatfish which found that individuals acclimated to light substrate preferred light backgrounds, while those acclimated to dark substrate showed no preference (Ryer et al., 2008). The preference for darker backgrounds in this study is not surprising given that camouflage was much better on the dark backgrounds than on the lighter ones. Poor camouflage on light coloured substrates, in combination with more reflected light, means they therefore carry a greater predation risk. For instance, Endler (1987) showed that predation risk in guppies is greatest at high light levels, presumably due to the greater contrast between the prey and its background making it easier for predators to detect them (e.g. Strand et al., 2007).

Although not as strong as the preference for black, fish acclimated to sand were found to have a preference for that background while fish acclimated to the rock background displayed no preference. This apparent difference in preference was not however statistically significant. This is nonetheless interesting because rock gobies were more camouflaged on the sand background thus a preference for this colour may have been expected. In contrast camouflage on the rock background was not as good which would explain why the rock fish showed no preference at all for this background. Overall the findings of the choice experiments support the suggestion that camouflage rather than familiarity with the background is controlling decision making because fish would be expected to have a preference for the background they had acclimated too if familiarity with the background was controlling decision making.

It is possible, though not strictly necessary, that at some level rock gobies may be aware of their own conspicuousness on different backgrounds. For instance, guppies have been found to prefer shoals with fish that match their own colour more than those that do not

(Rodgers et al., 2013) suggesting that they must have some awareness of their own colour. Hermit crabs for instance have been shown to be aware of their current conspicuousness and have a longer startle response when they are more conspicuous (Briffa and Twyman, 2011). Zebrafish are able to learn the phenotype of conspecifics, though they are not directly aware of their own phenotype (Engeszer et al., 2014).

It has been suggested that a preference for one substrate over another could be detrimental to survival as predators would learn to search for prey on their preferred background (Allen et al., 2010). However, a lack of background preference to reduce predator learning needs to be balanced with a preference for substrates on which the prey is able to camouflage best as choosing a mismatching background would also carry a high predation risk (Husak et al., 2006; Stuart-Fox et al., 2003). The strength of a preference for a particular background exhibited by any given species may therefore be expected to depend on the ability of the animal to change colour and pattern to match different substrates. This is because species with better dynamic background matching ability may evolve a weaker preference compared to species with a more limited colour and pattern changing ability (e.g. Allen et al., 2010; Tyrie et al., 2015).

Here colour change has been discussed in terms of crypsis; however it may also function for reasons other than camouflage. The Australian giant cuttlefish (*Sepia apama*) for instance changes colour to match its background for camouflage but also to increase conspicuousness during signalling (Zylinski et al., 2011). Moreover, in chameleons there is good evidence to suggest that colour change primarily evolved as a strategy to facilitate social signalling rather than a means of avoiding predators (Stuart-Fox and Moussalli, 2008). Colour change is also important for thermoregulation in many terrestrial species (Stuart-Fox and Moussalli, 2009). Since rockpools are often very shallow body temperature may very well be affected by direct radiation from the sun, as well as ambient water temperature. It remains to be seen if colour change in rockpool fish, such as the rock goby, functions for roles other than camouflage.

The findings of this chapter support the results of previous research on small rockpool fish (i.e. Stevens et al., 2014a) and demonstrate that gobies are able to achieve a much greater level of camouflage on natural colours compared to artificial colours that do not represent those found within their natural habitat. Furthermore, the finding show that a fish's ability to

match different backgrounds is not unbounded and behavioural background choice may also play an important role in achieving camouflage in rockpools. It is plausible that other fish species may also be better at matching certain natural colours more than others and that this difference in matching ability may be the result of asymmetries in selection pressure to match different colours. Furthermore, like rock gobies, other colour changing species may make up for these short falls in colour changing ability by exhibiting some degree of behavioural background matching.

Chapter 3: Rockpool gobies change the expression of their body pattern in response to changes in background markings

Photo credit: Sam Smithers

Abstract

There are numerous different camouflage strategies in nature that help prevent predators from detecting or recognising potential prey. While many animals have fixed body patterns that camouflage them against their background, others are capable of actively changing their body pattern to enable camouflage on a potentially wide range of background types. Such dynamic camouflage may be particularly advantageous for species inhabiting the intertidal zone, which tends to be highly heterogeneous, and where fish may be exposed to both terrestrial and marine predators depending on tidal level. Surprisingly, pattern change for camouflage is poorly studied in species inhabiting this zone. The rock goby (*Gobius paganellus*) is a common rockpool fish that has previously been used as a model species for studying rapid colour change in this environment. It is capable of rapidly (within one minute) changing its colour and luminance (perceived lightness) for camouflage, however nothing is known about the ability of rockpool fish to change their body pattern. In this chapter I used digital image analysis and a model of predator vision to first determine if rock gobies change their body pattern in response to their visual background, and then to explore how the spatial frequency of more natural backgrounds influences pattern change. Rock gobies rapidly changed their body pattern when placed on a checkerboard with squares measuring either 1 x 1 mm or 5 x 5 mm. When placed on controlled natural looking backgrounds, sand (high spatial frequency markings) elicited a greater change in pattern than backgrounds consisting of gravel, stones, or a mixture of all three substrate types. With the exception of individuals larger than ~70 mm in length, the majority of fish showed little, to no, improvement in background matching over time. Interestingly, the markings elicited by rock gobies are characteristic of disruptive coloration. I therefore put forward the suggestion that the body pattern expressed by rock gobies, and potentially other rockpool fish, may function primarily through disruptive camouflage, rather than background matching.

Introduction

Many animals use camouflage to conceal themselves from potential predators and there are countless camouflage strategies which have evolved in nature (Cott, 1940; Stevens and Merilaita, 2009a; Thayer, 1909). Background matching (e.g. Endler, 1984), disruptive coloration (e.g. Cuthill et al., 2005), countershading (e.g. Rowland et al., 2008), and masquerade (e.g. Skelhorn et al., 2010) are just a few examples of the many different camouflage strategies in nature. All of the aforementioned strategies, except masquerade, primarily prevent detection by predators and are collectively referred to as crypsis (Stevens and Merilaita, 2009a).

By far the most common form of crypsis is background matching, which is often fundamental in other camouflage strategies such as disruptive coloration (Stevens and Merilaita, 2009b). Background matching is when the appearance of an animal generally matches the colour, lightness and pattern of one or several background types (Stevens and Merilaita, 2009a). Based on this definition there are three main components that contribute to background matching, and indeed crypsis in general; an animal's colour, luminance (perceived lightness), and body pattern.

Many animals have evolved a fixed body pattern which allows them to camouflage themselves against their background (Marshall and Gluckman, 2015). However, the effectiveness of fixed patterns is limited to situations where prey is viewed against either a specific or a limited range of backgrounds. Many species with a fixed coloured pattern have therefore been shown to exhibit some form of behavioural camouflage whereby they actively select backgrounds against which they are most camouflaged (Kang et al., 2012, 2013; Kettlewell and Conn, 1977). For example two species of moth, *Hypomecis roboraria* and *Jankowskia fuscaria*, are known to orientate their bodies so their body markings are in line with the pattern of the tree bark they have settled on (Kang et al., 2012). Other animals with fixed patterns may have evolved a compromise pattern to increase camouflage on a variety of background types rather than specialise on one particular background (Houston et al., 2007; Merilaita et al., 1999, 2001). However, some species have gone one step further and have evolved the ability to change their coloration and body pattern in response to their background (Hanlon and Messenger, 1988; Ramachandran et al., 1996; Stuart-Fox and Moussalli, 2009; Watson et al., 2014). For instance, cuttlefish are able to rapidly change their

body pattern in response to changes in the size, colour and composition of their visual background (Barbosa et al., 2008b; Mäthger et al., 2007) and so improve camouflage in the eyes of their predators (Chiao et al., 2011). Flatfish are also well known for their ability to change body pattern in response to both natural and artificial backgrounds (Fujimoto et al., 1991; Healey, 1999; Kelman et al., 2006; Ramachandran et al., 1996; Sumner, 1911).

The above dynamic camouflage would be expected to provide a major survival advantage in heterogeneous habitats such as rocky shores where substrate type can vary substantially and where a range of different background patterns can exist within a very small area. Species inhabiting the rocky shore are often exposed to a range of different predators depending on tidal level, and the action of waves and currents can force animals onto many different background types. Common rockpool species from a variety of taxa have been found to change colour for camouflage (Fries, 1942; Keeble and Gamble, 1899; Stevens et al., 2014a, 2014b). One such species is the rock goby (*Gobius paganellus*) which has been shown to rapidly change its luminance and colour for camouflage (Stevens et al., 2014a).

Stevens et al., (2014a) tested rock gobies on red and blue artificial backgrounds and found that gobies became redder when placed on red, and greyer when placed on blue. Similarly in Chapter 2 fish were tested on more natural coloured backgrounds that matched either the colour of sand or green algae covered rocks. Fish on the sand coloured background became redder than those on the green rock background. In another experiment, Stevens et al., (2014a) found that rock gobies tested on either black or white decreased or increased their overall luminance respectively. The second experiment in chapter 2 expanded on this by testing rock gobies on a range of background brightness from black to white. As expected, overall luminance was found to increase with increasing background brightness. Interestingly the fish were always darker than their background even when they were capable of achieving a greater luminance such as when they were on the dark grey background. It was suggested in Chapter 2 (as well as in Stevens et al., (2014a)) that this could be due to the fact many individuals had body patterns that were darker than the rest of the body thus lowering the overall luminance of the fish. These markings are likely to play a role in camouflage and have been previously likened to disruptive coloration (Stevens et al., 2014a).

As discussed above, species such as flatfish which are able to change colour and luminance are also able to change their body pattern to better match their background

(Healey, 1999; Kelman et al., 2006; Ramachandran et al., 1996). However, beyond isolated studies such as Allen et al. (2015), which investigated adaptive pattern change in the slender filefish (*Monacanthus tuckeri*), few studies have looked at changes in pattern for camouflage outside flatfish and cephalopods, and fewer still have looked at intertidal species that are exposed to both aquatic and terrestrial predators. Gobiid gobies constitute the largest family of marine fish in the world (Helfman et al., 2009), and as such make an ideal model system for studying colour change for camouflage.

In this chapter I tested the ability of the rock goby, which is an abundant intertidal species around the UK (Dunne, 1978; Miller, 1961), to change body pattern in response to different backgrounds. Digital image analysis and a model of predator vision were used to quantify changes in body pattern, as per previously outlined methods. The study consisted of two experiments. The aim of the first experiment was to quantify whether or not rock gobies change their body pattern in response to their background. To test this I used different sized black and white checkerboards similar to those used in classic experiments on cuttlefish and flatfish (e.g. Ramachandran et al., 1996; Barbosa et al., 2008b). Studies on cuttlefish have shown that the spatial frequency (marking/object size) of the background affects their body pattern (Kelman et al., 2007, 2008). The second experiment therefore aimed to determine if, and how, pattern change in gobies is influenced by the spatial frequency of natural backgrounds. For this, grey-scale images of different sized black and white substrates were used. Grey-scaled images were used as this allowed us to test the effect of semi natural backgrounds with different spatial frequencies while keeping all other information about the background constant (e.g. achromatic, chromatic, and textural information were all controlled).

Methods

Preliminary experiment

Initial evidence of pattern change was established during field observations prior to the main study. Experiment 1 followed previous studies on cuttlefish and flatfish which used black and white checkerboard backgrounds to investigate how the animals changed their body pattern in response to different check sizes (e.g. Ramachandran et al., 1996; Chiao and Hanlon, 2001; Barbosa et al., 2008b). In order to choose the optimum check sizes (i.e. check sizes that elicited the greatest change in body pattern) to use in experiment 1 a preliminary experiment was carried out. This experiment was carried out in a 400 x 300 x 65 mm plastic tray that was divided into four sections each containing a different sized checkerboard (see below for details about background creation and experimental setup). The four check sizes used were: 1 x 1 mm, 5 x 5 mm, 10 x 10 mm and 20 x 20 mm. The general procedure was similar to that described below whereby fish were placed on the different backgrounds and photographed to find out if any of the backgrounds elicited a change in the fish's body pattern. The photos were used to subjectively choose the best backgrounds to use for experiment 1. Quantitative image analysis was not carried out for the preliminary experiment.

Generating the experimental backgrounds

Experiment 1

For experiment 1, two experimental backgrounds were created using different sized black and white checkerboards. The backgrounds were generated in the graphics program inkscape v0.48 whereby a RGB value of 0:0:0 was used to form the black squares and the white paper formed the white squares. One background had squares which measured 1 x 1 mm (small checkerboard) and the other had squares measuring 5 x 5 mm (large checkboard). These two sizes were chosen because they elicited the most noticeable change in body pattern in the preliminary study.

An intermediate grey was used for the starting background on which all fish were placed before starting the experiment. This was generated by creating a grid of grey squares starting with RGB values of 0:0:0 (black) and increasing in increments of 2 all the way to RGB values of 255:255:255 (white). The grid was then printed on waterproof paper (Xerox Premium NeverTear) using a Hewlett Packard LaserJet 500 color M551 PCL6 printer before

being photographed using a Nikon D7000 digital camera (see section below on image analysis). A black and white Spectralon (Labsphere) reflectance standard with a scale bar (see section on image analysis) was included in the photograph. An Iwasaki eyeColour MT70D E27 6500K arc lamp which had had the UV/IR protective filter removed was used as the light source for these photos. The grid was not photographed in ultraviolet (UV) because it is not possible to print in UV. The image of the grid was processed in a similar way to that described in the section below on image analysis. Ideally it would have been desirable to map the images to goby vision but there is insufficient data available to be able to do this. Instead the values for each of the camera's image channels (longwave (LW), mediumwave (MW), and shortwave (SW)) were used, whereby a value of 65535 on a 16-bit scale is equal to 100% reflectance (Stevens et al., 2007; Troscianko and Stevens, 2015). The green channel was used as the measure of luminance in accordance with previous work that used similar techniques to those described here (Spottiswoode and Stevens, 2011; Stevens et al., 2013). The grey, which had a reflectance value half way between the black and white, was chosen to form the starting background. The actual reflectance for the intermediate grey was ~49% since the actual reflectance of the black and white squares were ~8% and ~90% respectively.

Experiment 2

Four backgrounds were made from equal proportions of black and white aquarium substrates of different sizes. These backgrounds were sand (fine substrate size), gravel (medium substrate size), stones (large substrate size) and a mixture of all three. For the sand background 500 ml of white sand (Pettex Roman Gravel White Quartz Sand) and 500 ml of black sand (Pettex Roman Gravel Black Sand), both < 1 mm in diameter, were mixed together in a 350 x 250 x 50 mm white tray. A small amount of water was added to the sand during mixing to improve the contrast between the black and white particles and to help ensure an even pattern across the whole tray. For the medium sized gravel substrate 500 ml of white gravel (Classica White Aquarium Fish Tank Gravel) and 500 ml of black (Classica Black Aquarium Fish Tank Gravel) were mixed together so that the bottom of the tray was not visible though the gravel. The gravel measured between 5-8 mm in diameter. For the large stone substrate, black and white pebbles (shop brought polished river pebbles) were mixed together in equal amounts so that the bottom of the tray was completely covered. The pebbles measured between 20-40 mm in diameter. For the mixed background I used approximately 500 ml of each type of sand, 400 ml of each type of gravel and ~200 ml of each of the pebbles (these amounts were used so that the surface area of substrate type was

approximately equal when mixed together). These were then mixed together by hand in a white tray so that each substrate size randomly covered approximately one third of the surface area of the tray, and there was an equal amount of black and white of each substrate.

Next, the contents of each of the trays were photographed outside under natural daylight. Each tray was photographed at least five times with the substrate being randomly remixed between each photo. One image of each substrate type was used to form the experimental background for each treatment for all trials. For this I subjectively selected the photo in which the black and white substrates were distributed equally throughout the image, and there was no clumping of either colour in one area of the image. Each image was opened in Image J as a RAW file and then converted into a grey-scaled 8-bit JPEG file. Each image was then printed at the same scale as the original set up (i.e. a pebble which measured 30 mm will measure the same size in the printed image). In order to ensure that the overall brightness was the same across all backgrounds the four printed images were photographed outside and the brightness of each calculated as described above. The overall brightness of the darkest photos was artificially increased using the 'brightness/contrast' tool in Image J. These new images were then printed and photographed as before and the overall brightness recalculated. This process was repeated until the overall brightness of the four backgrounds was within 2% of one another. The starting background was created in much the same way as in experiment 1, whereby a grey of the same brightness as the experimental backgrounds was chosen from a printed grid.

Experimental set up.

The experiments were carried out in a 400 x 300 x 65 mm grey plastic tray. The tray was divided into four separate sections using 3 mm thick acrylic walls that were fixed in place using aquarium safe silicone adhesive. The four sections were in turn split in half by removable 2 mm thick acrylic dividers, each being held in place by transparent slide binders that were glued to the walls using the silicone adhesive. Each of the eight compartments measured approximately 85 x 13 mm. In both experiments the bottom and sides of the four middle compartments were covered with the starting grey while the four outside compartments were covered with either the small or large checkerboard in experiment 1, or the sand, gravel, stones or mixed grey-scaled backgrounds in experiment 2. The backgrounds were stuck to the sides of the tray as well as the bottom as studies have shown that both vertical and horizontal features influence pattern change in animals such as cuttlefish

(Barbosa et al., 2008a; Ulmer et al., 2013). The experimental setup and backgrounds for experiment 1 and 2 are shown in Figures 3.1 and 3.2 respectively. The tray was filled with fresh seawater to a depth of approximately 20 mm. Fresh seawater was used for each fish and the fixed acrylic walls prevented the flow of water between the four sections.

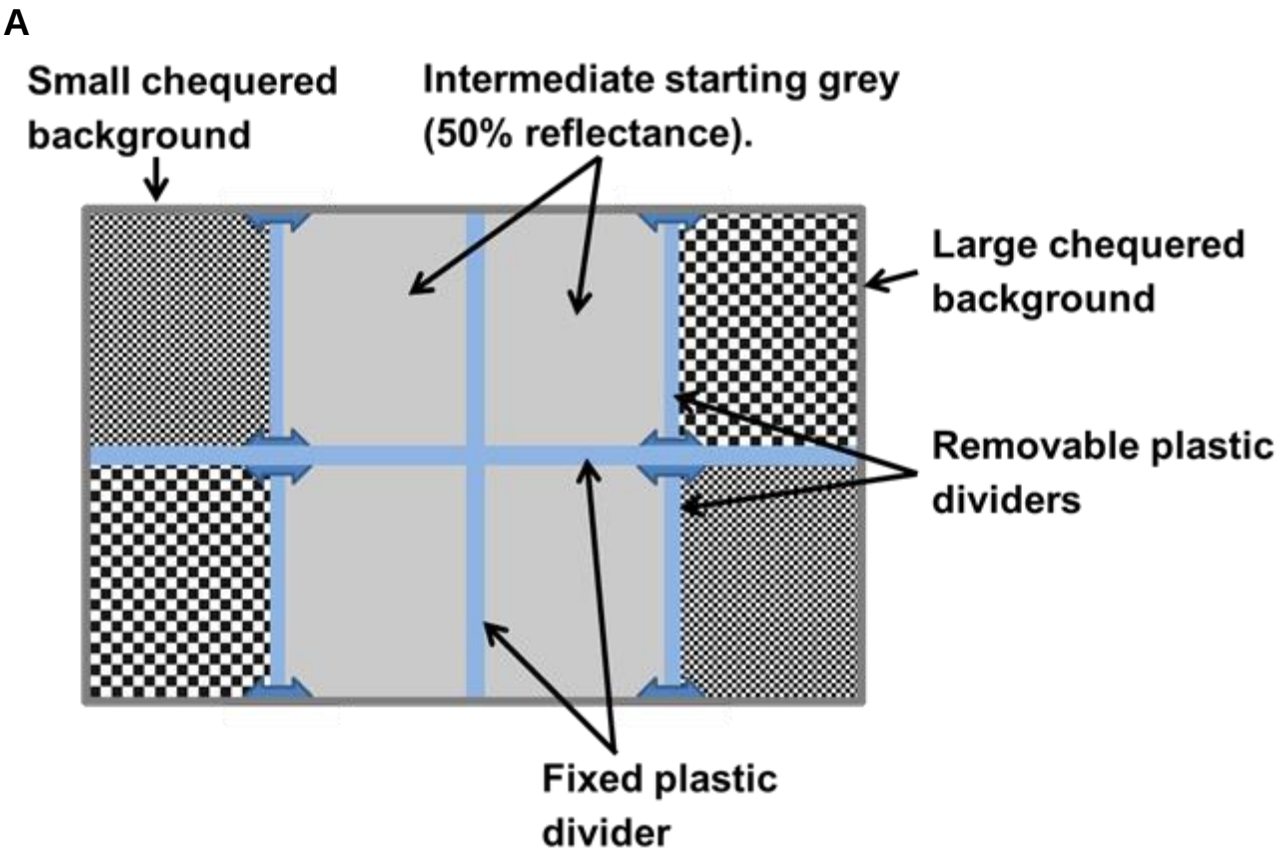

Figure 3.1: Experimental tray used for experiment 1. (A) Diagram showing the experimental backgrounds (not to scale) and the design of the tray used in experiment 1, and (B) final tray with two of the sliding dividers removed. Photo credit: Sam Smithers

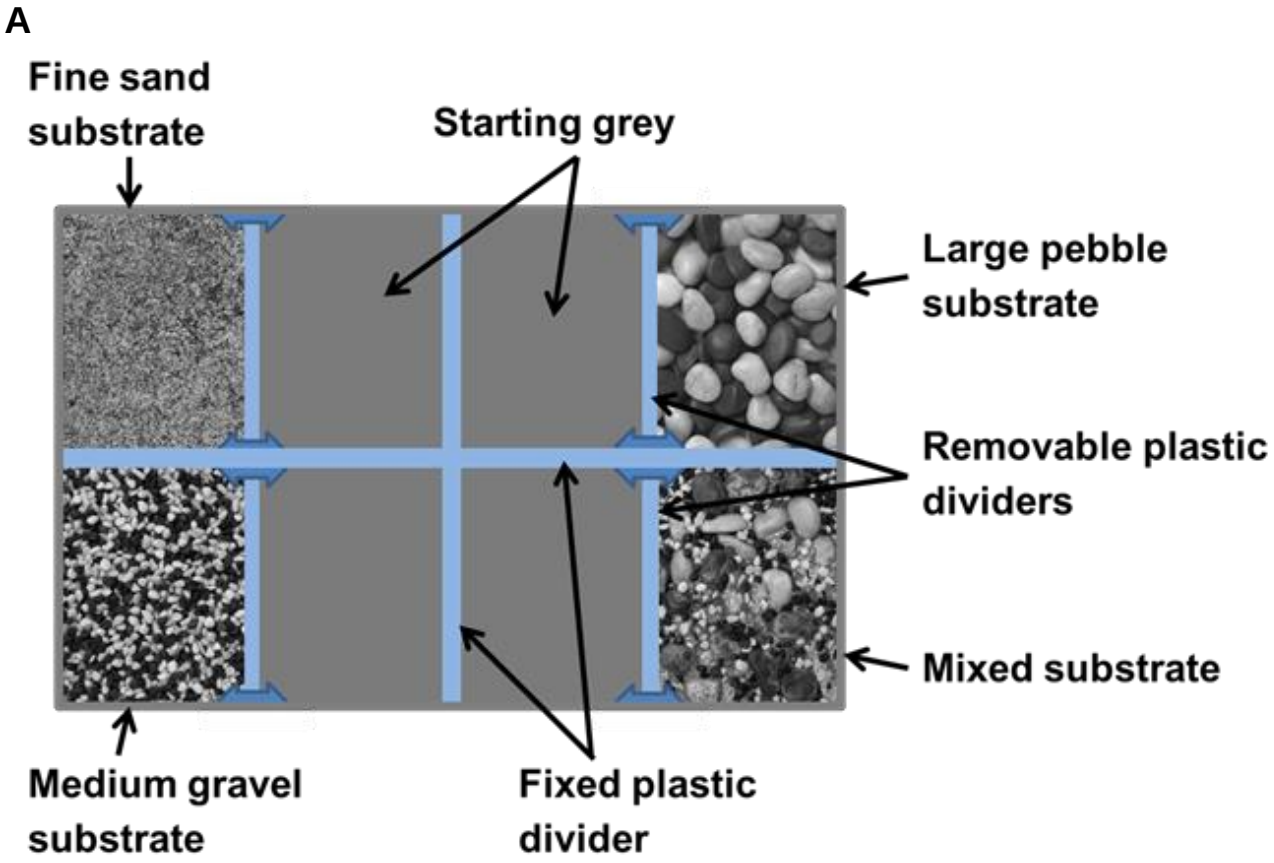

Figure 3.2: Experimental tray used for experiment 2. (A) Diagram showing the experimental backgrounds (not to scale) and the design of the tray used in experiment 1, and (B) final tray photographed during one of the trials. Photo credit: Sam Smithers

Experimental procedure

The experiments were carried out in situ on Gyllyngvase beach, Falmouth, Cornwall, UK (50° 8’933.46900”N, -005° 04’907.97160”W) between the start of May and end of June 2015. Fish were collected by hand and dip net from rock pools and placed in a grey bucket containing fresh seawater. A total of 40 fish were tested in experiment 1 and 80 fish in experiment 2 (20 individuals per background). All work was conducted under approval from the University of Exeter Biosciences ethics committee (application 2015/739). Gyllyngvase beach is public land and no further licences or permits were needed. All individuals were returned unharmed to their original rockpool area after being tested. Rock gobies are not an endangered or protected species.

Individuals were tested in size matched blocks in which fish were tested simultaneously ± 15 min to ensure any differences in pattern change between treatments were not the result of testing fish on different days, or at different times of day. For experiment 1 there were two fish in each block, while for experiment 2 there were four fish in each block. Before starting the experiment, each fish was first placed in the grey starting background and allowed to acclimatise for a minimum of 20 min in experiment 1, and 15 min in experiment 2. This was done to reduce individual differences between fish and to ensure that all fish acclimated to the same background before starting the experiment. This was important because the fish had been collected from different rockpools that often consisted of very different substrate types. Following this the fish were photographed in both visible and UV light and then immediately moved across to the experimental background by lifting the removable divider that separated the two sections. Each fish was then photographed at intervals of approximately 1, 5, and 30 min in experiment 1, and at 15 min in experiment 2. To control for differences in fish size between blocks and the effect it may have had on pattern change each fish was measured (excluding the tail) before being released.

Image analysis

All photographs were taken using a Nikon D7000 digital camera that had undergone a quartz conversion to enable photos to be taken in both visible and UV light (Advanced Camera Services, Norfolk, UK), and fitted with a Nikon 105 mm Nikkor lens. All photos were taken in RAW format with manual white balance and fixed aperture and ISO settings using manual focus. The lens was refocused between the visible and UV photos to maintain the sharpness

of each image. The human visible photos were taken using a UV/infrared (IR) blocking filter which transmits wavelengths of 400-700 nm (Baader UV/IR Cut/L Filter). The UV photos were taken using a UV pass and IR blocking filter which transmits wavelengths between 300 and 400 nm (Baader U filter). A custom made filter slider was used to quickly move between the two filters. Because overexposed photos cannot be used for image analysis bracketing (whereby three photos are taken at different shutter speeds) was used to ensure that at least one visible and one UV photo was correctly exposed. A black and white Spectralon reflectance standard (made from 10 x 10 mm sections of zenith diffuse sintered PTFE sheet, Labsphere), calibrated to reflect 8.3% and 94.7% of all wavelengths respectively, with a scale bar was included in all photos taken. It was important to ensure that the standard was viewed under the same light conditions as the fish. For this purpose the standard was placed in a custom made waterproof box which was positioned next to the fish in all photos. The box was made out of clear plastic which allowed both visible and UV light to pass through. A ring of aquarium safe lead inside the box was used to weigh the box down in the water next to the fish. A lid was placed over the box between photos to ensure the standard was not contaminated by dust or splashes of water. A tripod was used to position the camera directly above the fish and the standard at a height of approximately 50-70 cm. It was important to ensure that light levels were even across the whole tray and reflectance from the water surface was minimal. To this end a black and silver photographic umbrella (Neewer, Guangdong, China) was used to shade the trays from direct sunlight.

For each time point one visible and one UV photo with the correct exposure (i.e. the image with the highest exposure without being over exposed) was chosen for image analysis using the RGB histograms provided in the program RawTherapee v4.1.80. Image and pattern analysis was conducted using the 'Multispectral Image Calibration and Analysis Toolbox' by Troscianko and Stevens (2015). The visible and UV photos were first combined into a single multispectral image consisting of information from both the visible and UV channels. In order to correct for the non-linear responses in image values that are produced by cameras in response to changes in light levels, all of the photos were linearised with regards to light intensity (Stevens et al., 2007). Following linearisation, image values were equalised with regards to the 8.3% and 94.7% grey standards, and each of the image channels (LW, MW, SW and UV) were scaled to reflectance whereby a value of 65535 on a 16-bit scale is equal to 100% reflectance (Stevens et al., 2007; Troscianko and Stevens, 2015). Next, a 20 mm scale bar was added to each multispectral image and the area of the fish's body (not including

the gills, eyes, or pectoral and caudal fins) was selected by hand and saved as a 'region of interest' (ROI).

Changes in pattern and camouflage were analysed with regards to the visual system of shorebirds, which are likely to be a key predator of rockpool fish at low tide (Stevens et al., 2014a). To do this I mapped the images to avian vision based on spectral sensitivity data from the peafowl (*Pavo cristatus*) (Hart, 2002) using a polynomial mapping technique to convert from camera to avian colour space under a D65 standard irradiance spectrum (Stevens et al., 2007; Troscianko and Stevens, 2015). The peafowl is often used as a model species for modelling birds that have a 'violet' sensitive (VS) visual system such as the majority shorebirds (Ödeen et al., 2009). In birds with a violet sensitive system the sensitivity of the UV cone type is shifted to slightly longer wavelengths than species which have an 'ultraviolet' sensitive visual system (Ödeen et al., 2009). It should however be noted that species within the violet group can still detect UV light. Gulls however are thought to have a UV visual system (Ödeen et al., 2009) but the differences in the perception between these two systems is likely to be small since both the backgrounds and the fish had relatively low levels of UV reflectance. In any case, the choice of a VS system based on the peafowl is most accurate for a shorebird visual sensitivity as most gulls are not considered to be shorebirds. Compared to modelling predicted cone catch values with reflectance spectra, this mapping technique is highly accurate, with very low levels of potential error and R^2 values for each channel from 0.96 to 0.98 between derived cone catch values based on spectrometry and cameras (Pike, 2011; Stevens and Cuthill, 2006; Troscianko and Stevens, 2015).

To analyse changes in pattern over time I used a 'granularity' analysis which has previously been used to study pattern in cuckoo eggs (Stoddard and Stevens, 2010) and shore crabs (Stevens et al., 2014b), and camouflage in cuttlefish (Barbosa et al., 2008b; Chiao et al., 2009). Granularity analysis is used to analyse the contribution that different marking sizes make to a given pattern (Stoddard and Stevens, 2010). It involves fast Fourier transforming each of the original calibrated multispectral images and applying 19 octavewide, isotropic band-pass filters to produce 19 images, referred to as 'granularity bands', each containing information at a different spatial scale. These filters function like a sieve starting with small markings (2 pixels) and increasing in size to larger markings (up to 1024 pixels) with a scale incrementing from 2 to the square root of 2 (Stevens et al., 2014b; Troscianko and Stevens, 2015). The 19 different granularity bands can be added together to give a close

approximation of the original unfiltered image without losing much information (Chiao et al., 2009; Stoddard and Stevens, 2010). Pattern analysis was conducted in Image J using the toolbox's 'Batch Multispectral Image Analysis' tool provided by Troscianko and Stevens (2015). All images were scaled to 37 px/mm.

The granularity bands were used to determine the relative contribution that different marking sizes make to the overall body pattern, and to quantify how the overall pattern changes over time once the fish had been exposed to the experimental backgrounds. Overall pattern 'energy' (sometimes referred to as 'power') was calculated for each granularity band as the sum of the squared pixel values in each image divided by the number of pixels in the image, with the actual scale being arbitrary (Stoddard and Stevens, 2010). The greater the pattern energy for any given granularity band the more dominant that particular marking size. The values of energy across all 19 band-pass filtered images produce a 'granularity spectrum' which can be plotted as energy versus pixels (marking size) (Stevens et al., 2014b).

To quantify changes in pattern over time, the granularity spectra of each fish at 0 min was compared with the spectra of the same individual at each of the other time points (1, 5, and 30 min for experiment 1, and 15 min for experiment 2) by calculating the sum of the absolute pattern energy difference (PED) between the two spectra at each spatial scale (Troscianko and Stevens, 2015). Noise in the system would not be expected to increase pattern difference unless that noise were biased. Any scale or contrast bias coming into the system would however be minimal. One potential source of noise would be pixel noise, which is very fine-scale, and ISO dependent. Since all photos were taken at the same ISO then any pixel noise will be very stable when measured across thousands/millions of pixels. Noise at larger scales is very unlikely because in order to get such noise large parts of the image (hundreds/thousands of pixels) would need to change shape or contrast substantially, which would probably make the image unintelligible. Any noise that does occur in an image when averaged across thousands (even millions) of pixels is unlikely to introduce systematic bias thus any difference in PED between images as a result of noise would be minimal. However, even if this noise were to exist to any meaningful degree, it would raise some parts of the energy spectrum and lower others. If the parts rise and fall around a mean with no bias, the average PED would be the same when any two energy spectra are compared (Jolyon Troscianko, personal communication).

To quantify changes in camouflage over time, the granularity spectra of the fish's background was compared with that of the fish at each time point. To obtain the granularity spectra of the background, each background was photographed in the tray (without water) and analysed in the same way as above. For the ROI the whole area of the background (minus that at the very edge of the image) was selected.

In addition to the PED, each granularity spectrum was used to calculate a range of information about the pattern of the fish. The predominant marking size in the pattern corresponded to the filter size containing the maximum value of energy in the spectrum. The higher the value for this filter size the larger the dominant marking size. The relative importance of this marking size to the overall pattern was determined by calculating the proportion of the total energy across all scales corresponding to the maximum energy (proportion energy). High proportion energy means that the pattern is dominated by that marking size, while low proportion energy indicates a more diverse pattern. As a measure of overall pattern contrast I used the total energy across all filter sizes which corresponds to the overall amplitude of the spectrum. The higher the total energy the more contrasting the markings are (Chiao et al., 2009; Stoddard and Stevens, 2010; Troscianko and Stevens, 2015).

Statistical analysis

When analysing the change in pattern over time, the values for the PED were log transformed. For change in pattern over time a PED of zero would be predicted if gobies do not change their body pattern. I therefore used one sample t-tests to test for a significant change in pattern between 0 min and each time point (1, 5, and 30 min for experiment 1, and 15 min for experiment 2) after the fish had been placed on the experimental background (i.e. does the PED at each time point differ from zero). To test whether background and fish size affected the change in pattern over time the values for PED were analysed using a general linear mixed effects model for experiment 1, and a general linear model for experiment 2. In both models test background and fish size were included as fixed factors. To test for a difference in PED between 1, 5, and 30 min in experiment 1 time point was included in the model as a fixed factor with fish identification (ID) as a random factor.

When analysing the difference in pattern between the fish and their background (i.e. change in the level of background matching over time) the values for the PED did not need transforming. General linear mixed effects models containing background, fish size, and time point as fixed factors, and fish ID as a random factor were used to analyse the change in camouflage over time for both experiments. I included all possible interactions in the models and used model simplification to test for significant interactions and fixed factors whereby models were fitted by maximum likelihood and compared with one another using a likelihood ratio test (LRT) for the general linear mixed effects models, and an F-test for the general linear models. For simplicity, in most cases only significant interactions are reported in the results. Formal statistical analysis was not carried out on the data for dominant marking size, proportion energy, or total energy. Instead these descriptive statistics were simply used to describe how the fish's pattern was changing over time. All statistical analysis and graphical modelling was carried out in R (R Core Team, 2014). The R package lme4 (Bates et al., 2014) was used for the general linear mixed effects models.

Results

Experiment 1

Change in pattern over time

For fish on both backgrounds there was a highly significant difference in pattern between 0 and 1 min (One Sample t-test: t= 55.83, df= 39, p<0.001; Figure 3.3a), 5 min (t= 56.7.81, df= 39, p<0.001), and 30 min (t= 67.77, df= 39, p<0.001). The greatest change in pattern occurred between 0 and 30 min, though it is however evident from Figure 3.3a that the majority of change in pattern occurred within the first minute of being placed on the experimental background. Indeed analysis using a general linear mixed effects model found no significant difference in the PED across the different time points (discounting 0 min which was used as the reference against which all other time points were compared) (likelihood ratio test: $chisq_2$=4.6, p=0.101). From Figure 3.3a it also appears that the small checkerboard background elicited a greater change in body pattern than the large checkerboard background (particularly at 5 min). However this difference was not significant ($chisq_1$=2.74, p=0.098), indicating that both check sizes elicited the same degree of pattern change. There was, however, a significant effect of fish size ($chisq_1$=7.98, p=0.005), whereby larger fish tended to show a greater PED than smaller fish (see Figure 3.3b). This trend is most apparent within the first 5 min of being moved to the checkerboard background. The exception is at 30 min for fish on the large checkerboard, which show no real effect of size. There also appears to be more variation in PED between fish within the first 5 min with less variation being observed between fish after they had been on the background for 30 min.

Change in camouflage over time

The difference in pattern between the fish and their background is shown in Figure 3.4a. There was a highly significant effect of check size ($chisq_1$=72.54, p<0.001), whereby fish were more camouflaged on the small checkerboard than on the large checkerboard. There was also a significant interaction between time and fish size ($chisq_3$=15.59, p=0.001; Figure 3.4b). This was the result of larger fish becoming more camouflaged over time (though this is not seen at 30 min for fish on the large checkerboard). However, for fish below ~60 mm in size there was very little, or no change, in camouflage over time meaning that the majority of the fish tested did not improve their camouflage over time.

Predominant marking size

The predominant marking size in the pattern corresponds to the filter size (granularity band) containing the maximum value of energy in the spectrum (Chiao et al., 2009; Stoddard and Stevens, 2010). Higher values correspond to larger marking sizes (low spatial frequency) while lower values correspond to smaller marking sizes (high spatial frequency).

Overall there was little change in the dominant marking size over time (Figure 3.5a). However, Figure 3.5a does suggest a slight shift in the dominant marking size to smaller markings for fish on the small checkerboard background. There are a number of outliers shown at 5 and 30 min which can be explained by taking into account fish size. Figure 3.5b suggests that as size increases there is an increase in dominant marking size after 5 min meaning that the dominant marking size of largest fish is larger at 5 and 30 min than at 0 or 1 min. There was however very little or no change in the dominant marking size for fish less than ~60 mm in size.

Pattern diversity

Proportion energy is the maximum energy divided by the summed energy (total energy) and is a measure of pattern diversity, or how much the pattern is dominated by one marking size (Chiao et al., 2009; Stoddard and Stevens, 2010). A high value of proportion energy means that the pattern is dominated by one marking size. Since the overall pattern was divided into 19 granularity bands the proportion energy would be predicted to be approximately 0.053 if all marking sizes contributed equally to the overall pattern.

From Figure 3.6a there appears to be an increase in proportion energy over time for fish tested on the small checkerboard indicating that the dominant marking size becomes more important (i.e. the overall body pattern becomes less diverse) over time. The degree to which the proportion energy changes with time depends on the size of the fish. For instance fish tested on the small checkerboard showed an increase in proportion energy over time, but this increase is reduced as fish size increases (Figure 3.6b). Conversely fish tested on the large checkerboard background appeared to show little or no change in proportion energy over time regardless of size. Interestingly, both groups appear to show an overall decrease in proportion energy as the size of the fish increases, suggesting that larger fish have more diverse patterns than smaller ones. It should be noted that in all but a few fish the proportion

energy is below 0.1 meaning that other marking sizes likely provide an important contribution to the overall body pattern.

Overall pattern contrast

Total energy is the energy summed across all 19 granularity bands and provides a measure of pattern contrast (Chiao et al., 2009; Stoddard and Stevens, 2010). The higher the value of the total energy the more contrasting the markings are.

Without taking size into account there is very little change in the total energy over time (Figure 3.7a). There are however a number of outliers which are indicative of a size effect. This is apparent in Figure 3.7b which shows that fish below ~60 mm in length showed little change in total energy over time when tested on the small checkerboard. Above this size the model predicts an increase in total energy over time as fish size increases, suggesting that larger fish developed a more contrasting pattern in response to the small checkerboard background. This trend coincides with observations of the fish made during the experiment. A similar, but albeit weaker, trend is also apparent for fish tested on the large checkerboard whereby there was no change in the total energy with time for fish below ~50-60 mm but above this size there is an increase in total energy over time as fish size increased, though this was not seen at 30 min. Figure 3.7b also suggests there is an overall increase in pattern contrast as size increases (shown by the increase in total energy with size at 0 min).

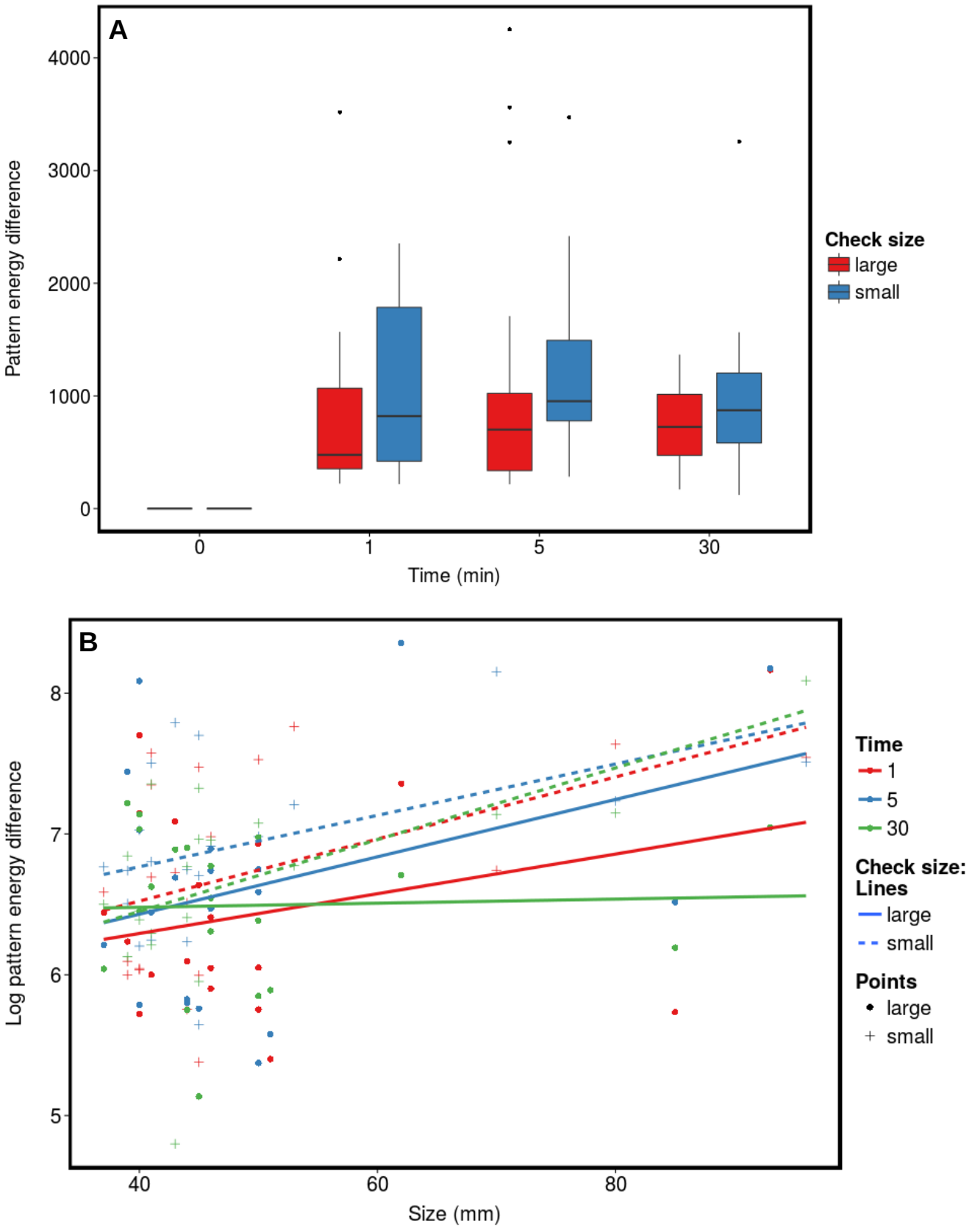

Figure 3.3: Change in pattern over time for fish tested on the small and large checkerboards in experiment 1. There was a significant change in pattern within 1 minute for gobies placed on both backgrounds. Overall, the larger the fish, the greater the change in pattern. (A) Pattern energy difference (PED) between the granularity spectra of the fish at the start of the experiment (0 min) and the granularity spectra of the fish at 1, 5, and 30 min. Graph shows medians plus inter-quartile range (IQR), whiskers are lowest and highest values that are within 1.5*IQR from the upper and lower quartiles, outliers are shown by dots. (B) PED between the granularity spectra of the fish at the start (0 min) and the granularity spectra of the fish at 1, 5, and 30 min against fish size. Lines show general linear models for fish on the two backgrounds at each time point.

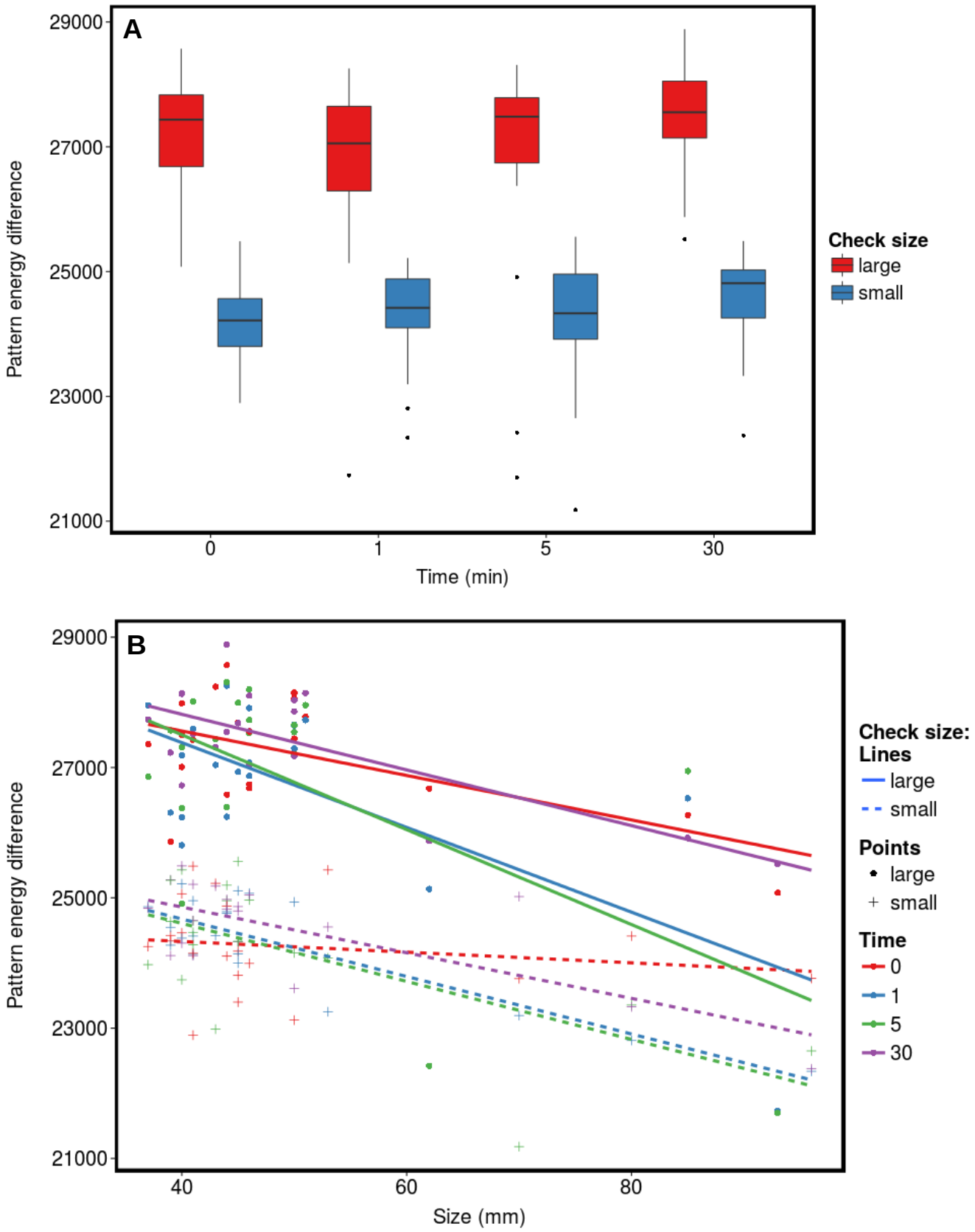

Figure 3.4: Change in camouflage over time for fish tested on the small and large checkerboards in experiment 1. Camouflage was significantly better on the small checkerboard than on the large checkerboard. There was a significant improvement in camouflage over time for fish over ~60 mm. (A) Pattern energy difference (PED) between the granularity spectres of the fish and the background it was placed on, at 0, 1, 5, and 30 min. Graph shows medians plus inter-quartile range (IQR), whiskers are lowest and highest values that are within 1.5*IQR from the upper and lower quartiles, outliers are shown by dots. (B) PED between the granularity spectra of the fish and its background at 0, 1, 5, and 30 min against fish size. Lines show general linear models for fish on the two backgrounds at each time point.

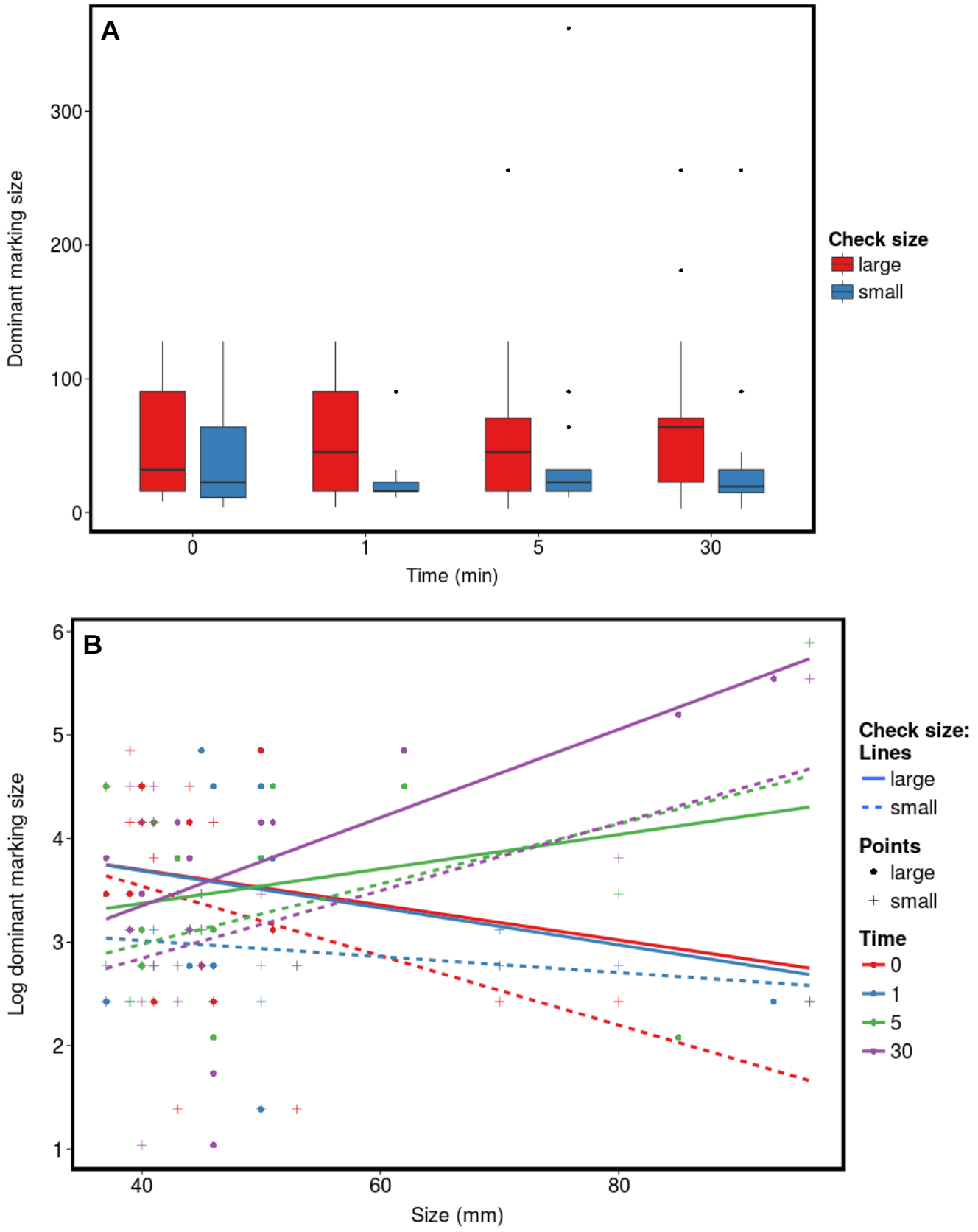

Figure 3.5: Change in dominant marking size over time for fish tested on the small and large checkerboards in experiment 1. Overall, there was little change in the most dominant marking size over time. The exception to this is seen in fish over ~60 mm that appear to show an increase in dominant marking size after 5 min. (A) Dominant marking size of fish at 0, 1, 5, and 30 min. Graph shows medians plus inter-quartile range (IQR), whiskers are lowest and highest values that are within 1.5*IQR from the upper and lower quartiles, outliers are shown by dots. (B) Dominant marking size of fish at 0, 1, 5, and 30 min against fish size. Lines show general linear models for fish on the two backgrounds at each time point.

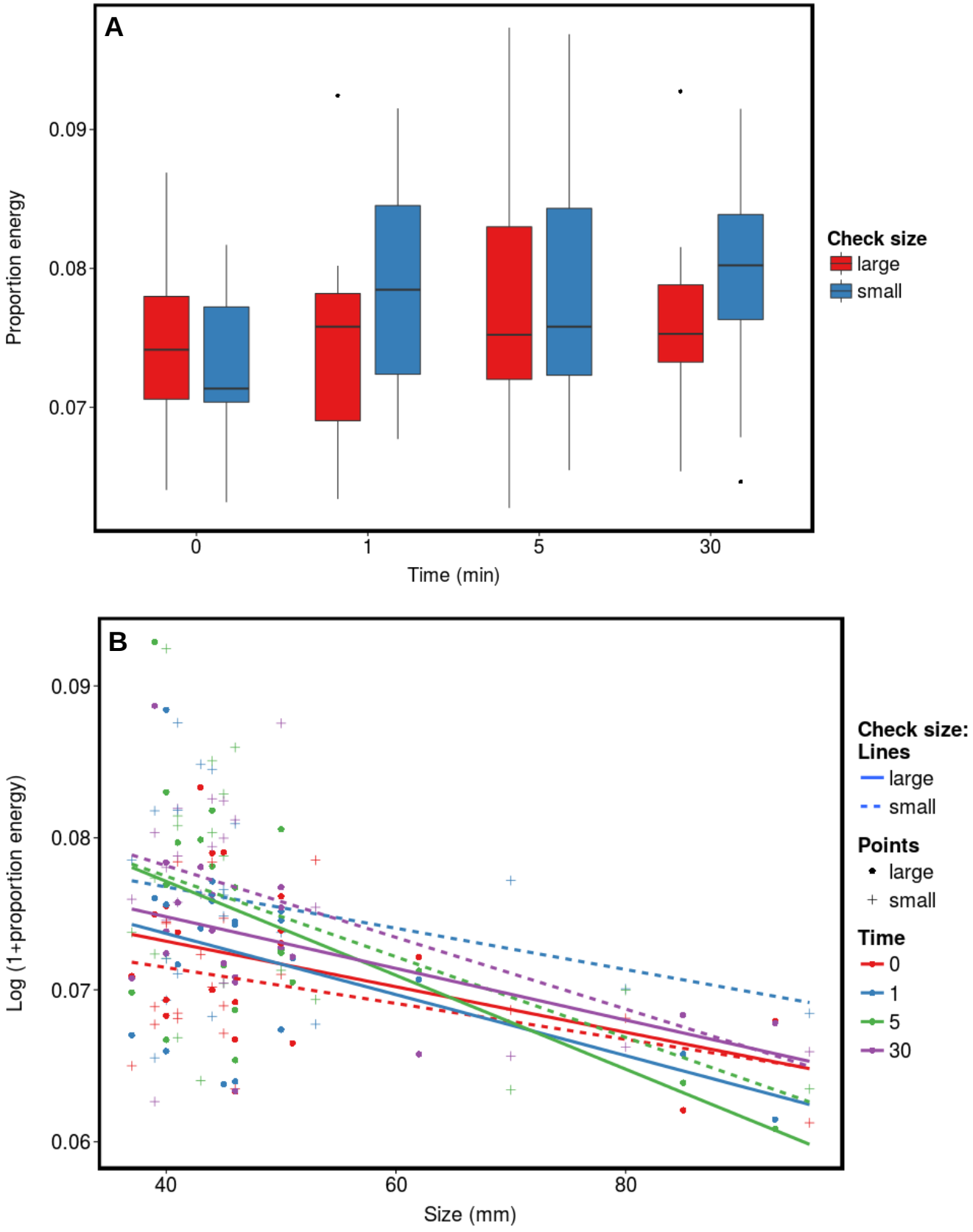

Figure 3.6: Change in pattern diversity, or the importance of the dominant marking size, over time for fish tested on the small and large checkerboards in experiment 1. There was a small increase in the relative importance of the dominant marking size over time for fish placed on the small checkerboard. There was an overall increase in pattern diversity with increasing fish size. (A) Pattern diversity of fish at 0, 1, 5, and 30 min. Graph shows medians plus inter-quartile range (IQR), whiskers are lowest and highest values that are within 1.5*IQR from the upper and lower quartiles, outliers are shown by dots. (B) Pattern diversity of fish at 0, 1, 5, and 30 min against fish size. Lines show general linear models for fish on the two backgrounds at each time point.

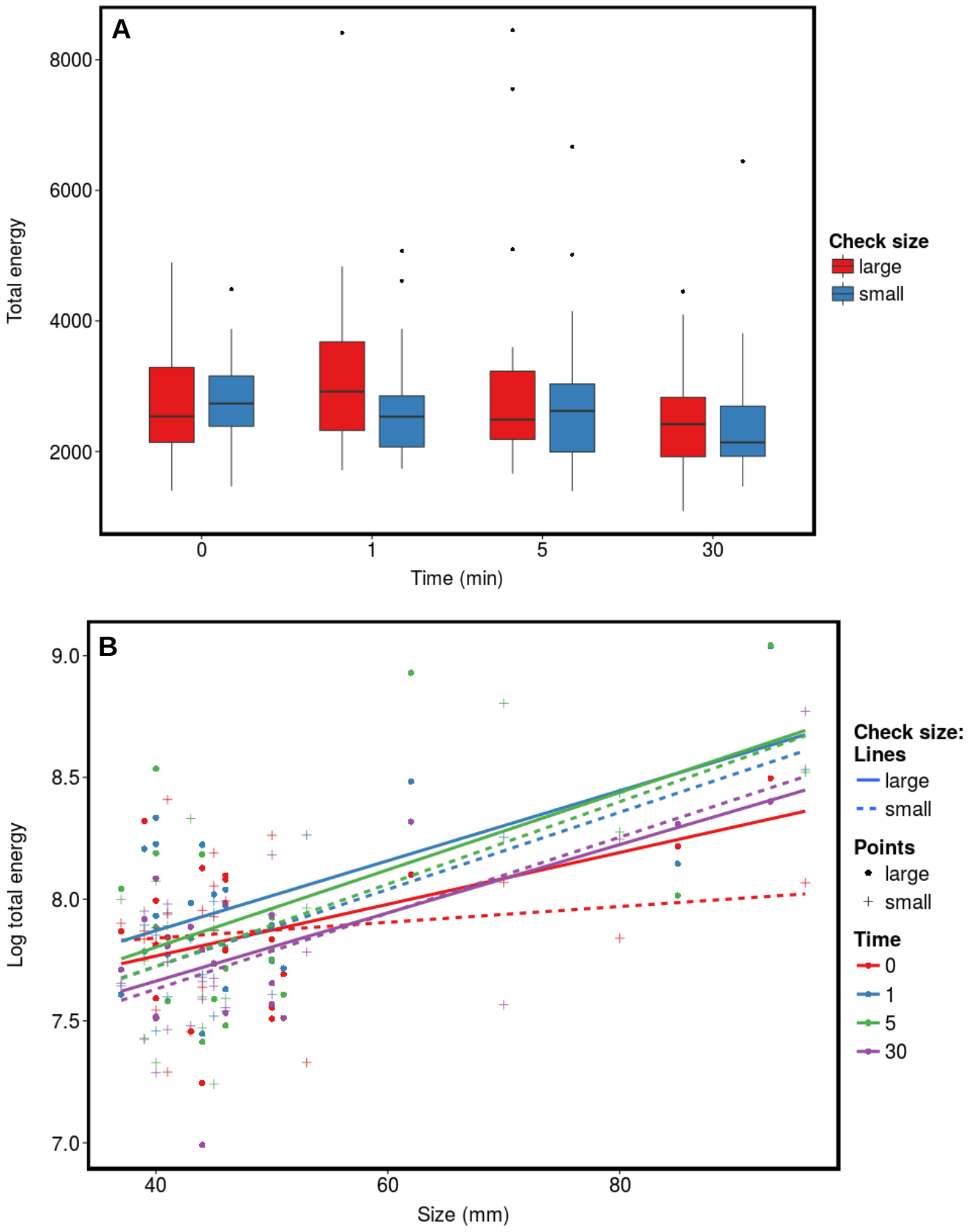

Figure 3.7: Change in pattern contrast over time for fish tested on the small and large checkerboards in experiment 1. There was little overall change in pattern contrast over time, although fish over ~70 mm did show a small increase in pattern contrast after 1 min. In general, larger fish tended to have more contrasting body patterns. (A) Pattern contrast of fish at 0, 1, 5, and 30 min. Graph shows medians plus inter-quartile range (IQR), whiskers are lowest and highest values that are within 1.5*IQR from the upper and lower quartiles, outliers are shown by dots. (B) Pattern contrast of fish at 0, 1, 5, and 30 min against fish size. Lines show general linear models for fish on the two backgrounds at each time point.

Experiment 2

Change in pattern over time

For fish on all four backgrounds there was a highly significant difference in pattern between 0 min and 15 min (One Sample t-test: t= 78.94, df= 79, p<0.001; Figure 3.8a). Statistical analysis using a general linear model found a significant effect of background, (F-test: $F_{3,75}$= 4.05, p=0.01), whereby the greatest PED was seen for fish tested on the sand background (see Figure 3.8a). The PED for fish tested on the gravel, stones and mixed backgrounds are very similar. There was also a highly significant effect of fish size ($F_{1,75}$= 14.37, p<0.001; Figure 3.8b). There is a similar trend to that seen in response to the checkerboard backgrounds in Experiment 1, whereby as fish size increases the PED also increases (though this trend was not seen in fish tested on the stones background). To test whether this trend was due in part to the influence of the few individuals above 70 mm the data was remodelled but with these data points removed. The same trend was seen and background and fish size were both significant when fish over 70 mm were removed from the model. The separate linear models shown in Figure 3.8b also support the deduction from Figure 3.8a that fish placed on the sand background had a greater PED compared to fish placed on the other backgrounds. Fish tested on the stone background showed no increase in PED with size. It is worth noting that overall the four substrates elicited less pattern change (a lower PED) than the two checkerboard backgrounds used in experiment 1.

Change in camouflage over time

The difference in pattern between the fish and their background is shown in Figure 3.9a. There was a significant three way interaction between background, size, and time (likelihood ratio test: $chisq_3$=13.69, p=0.004). This interaction indicates that the effect of size on the response to a background varies over time. For instance, Figure 3.9b indicates an effect of size over time for fish placed on the sand and mixed backgrounds whereby larger fish show a greater improvement in the level of camouflage after 15 min. Conversely, there appears to be no effect of size over time for fish placed on the gravel and stone background. This is supported by the fact that when the data points for the few fish over 70 mm in size were removed from the model the three way interaction was no longer significant ($chisq_2$=0.5, p=0.92). There was however a significant interaction between size and time ($chisq_1$=4.13, p=0.042), whereby there was a weak negative correlation between fish size and PED. This means that there was an overall improvement in camouflage as fish size increased (but this

increase was hidden on gravel and stones due to a few overly influential outlier). It should however be noted that any improvement in camouflage was small and may not necessarily effect the detectability of the fish in real terms. This is because even after 15 min the PED between the fish and its background was still very high. In addition the difference in camouflage between backgrounds remained highly significant when fish greater than 70 mm were excluded from the model ($chisq_3$=362.26, $p<0.001$). Fish were most camouflaged on the stone substrate and least camouflaged on the gravel. The PED on the sand and mixed substrates were very similar to one another with the level of camouflage being intermediate between that on the gravel and stone backgrounds.

Predominant marking size

As in the previous experiment there is little change in the dominant marking size over time (see Figure 3.10a), though there is a small shift in the medium for fish on the sand and gravel backgrounds towards larger markings. Figure 3.10b suggests that there is an increase in the dominant marking size over time as fish size increases. The same trend was still seen when the data points for the fish above 70 mm were removed, although it was much weaker and unlikely to amount to any noticeable difference in the most dominant marking size over time.

Pattern diversity

There was no change in proportion energy between 0 and 15 min (Figure 3.11a) for any of the backgrounds tested. Furthermore the relative importance of the dominant marking size can be considered the same on all four backgrounds. As was the case in the previous experiment there was an overall negative correlation between proportion energy and size, whereby the proportion energy decreased with increasing fish size (see Figure 3.11b). This trend did not change even after the data point for fish above 70 mm were removed from the models. The only exception was seen in fish on the gravel background, which showed no change in proportion energy with fish size at 15 min (but did at 0 min). This supports the suggestion that body pattern becomes more diverse as fish get larger.

Overall pattern contrast

There was an increase in pattern contrast between 0 and 15 min for fish tested on the sand background (see Figure 3.12a). There was also a far smaller increase in total energy with time for fish on both the gravel and the mixed backgrounds. Very little or no change in total energy is seen for fish tested on the stone background. Fish placed on the sand background

showed a strong trend for the total energy to increase with size at 15 min, but less so at 0 min (see Figure 3.12b). Similarly there is also an overall positive correlation between the total energy and fish size for the gravel, stone, and mixed backgrounds. Overall the effect of size is greatest at 15 min. It should also be noted that the models do not change if the data points for the largest individuals (i.e. fish over 70 mm) are removed from analysis. Therefore there is a strong trend for pattern contrast to increase as fish become larger.

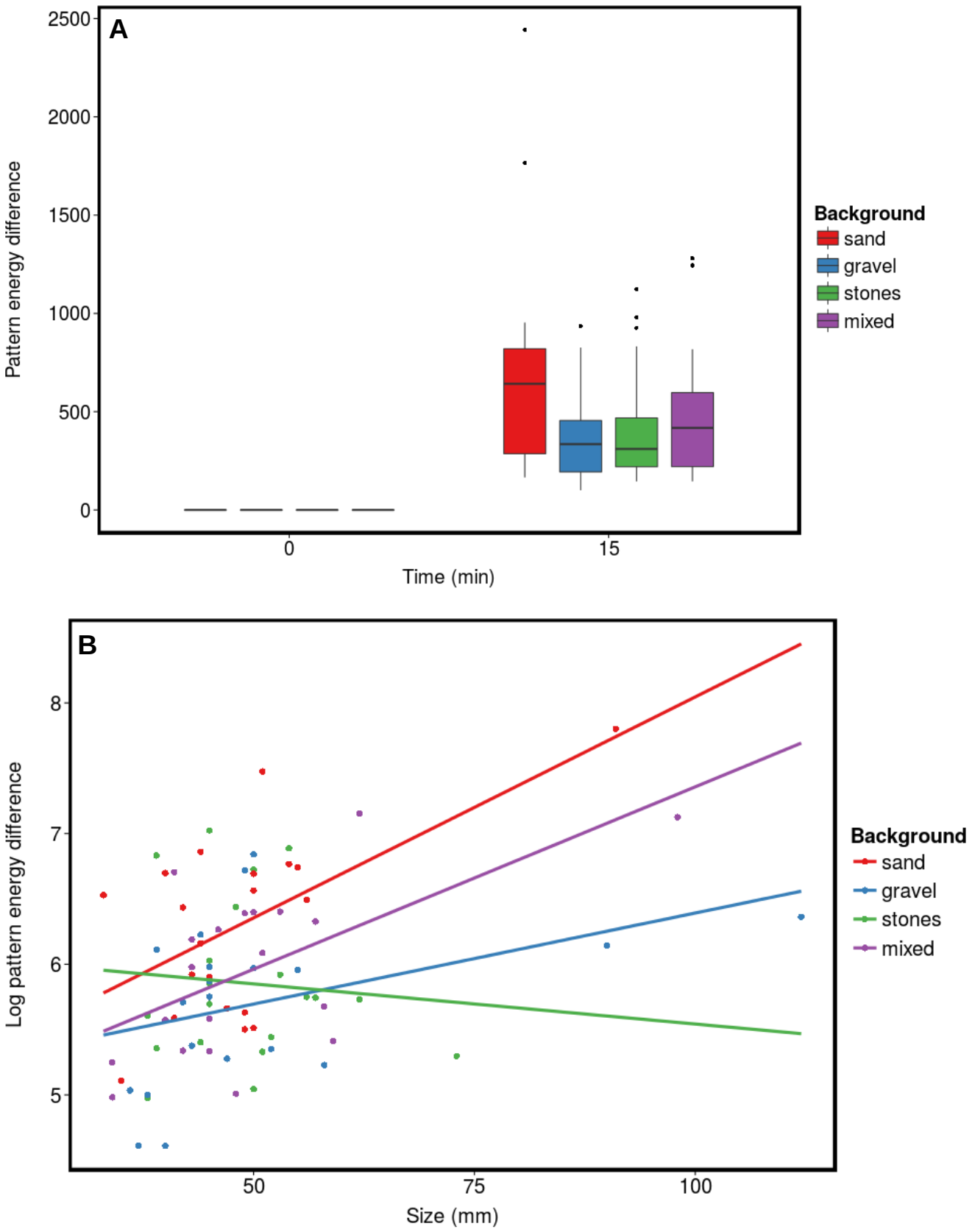

Figure 3.8: Change in pattern over time for fish tested on the sand, gravel, stone, and mixed substrate backgrounds in experiment 2. There was a significant change in pattern within 1 minute for gobies placed on all four backgrounds, with the greatest pattern change being seen in fish placed on the sand background. Overall, the larger the fish, the greater the change in pattern. (A) Pattern energy difference between the granularity spectra of the fish at the start of the experiment (0 min) and the granularity spectra of the fish at 15 min. Graph shows medians plus inter-quartile range (IQR), whiskers are lowest and highest values that are within 1.5*IQR from the upper and lower quartiles, outliers are shown by dots. (B) PED between the granularity spectra of the fish at the start (0 min) and the granularity spectra of the fish at 15 min against fish size. Lines show general linear models for fish on the four backgrounds at each time point.

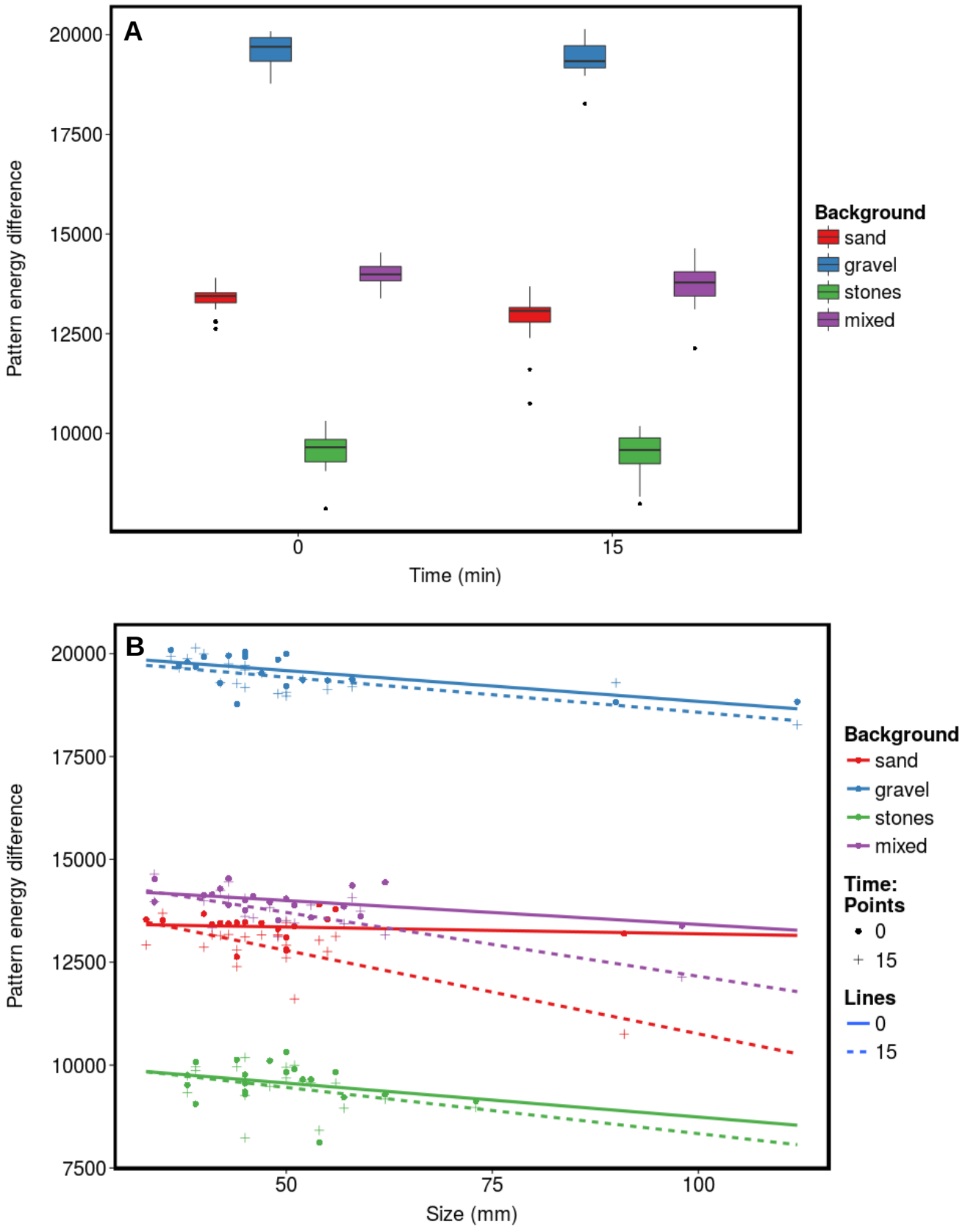

Figure 3.9: Change in camouflage over time for fish tested on the sand, gravel, stone, and mixed substrate backgrounds in experiment 2. Rock gobies were most camouflaged on the stones background and least camouflaged on the gravel background. There was a small overall improvement in camouflage over time with larger fish generally showing the greatest improvement. (A) Pattern energy difference (PED) between the granularity spectra of the fish and the background it was tested on, at 0, and 15 min. Graph shows medians plus inter-quartile range (IQR), whiskers are lowest and highest values that are within 1.5*IQR from the upper and lower quartiles, outliers are shown by dots. (B) PED between the granularity spectra of the fish and its background at 0, and 15 min against fish size. Lines show general linear models for fish on the four backgrounds at each time point.

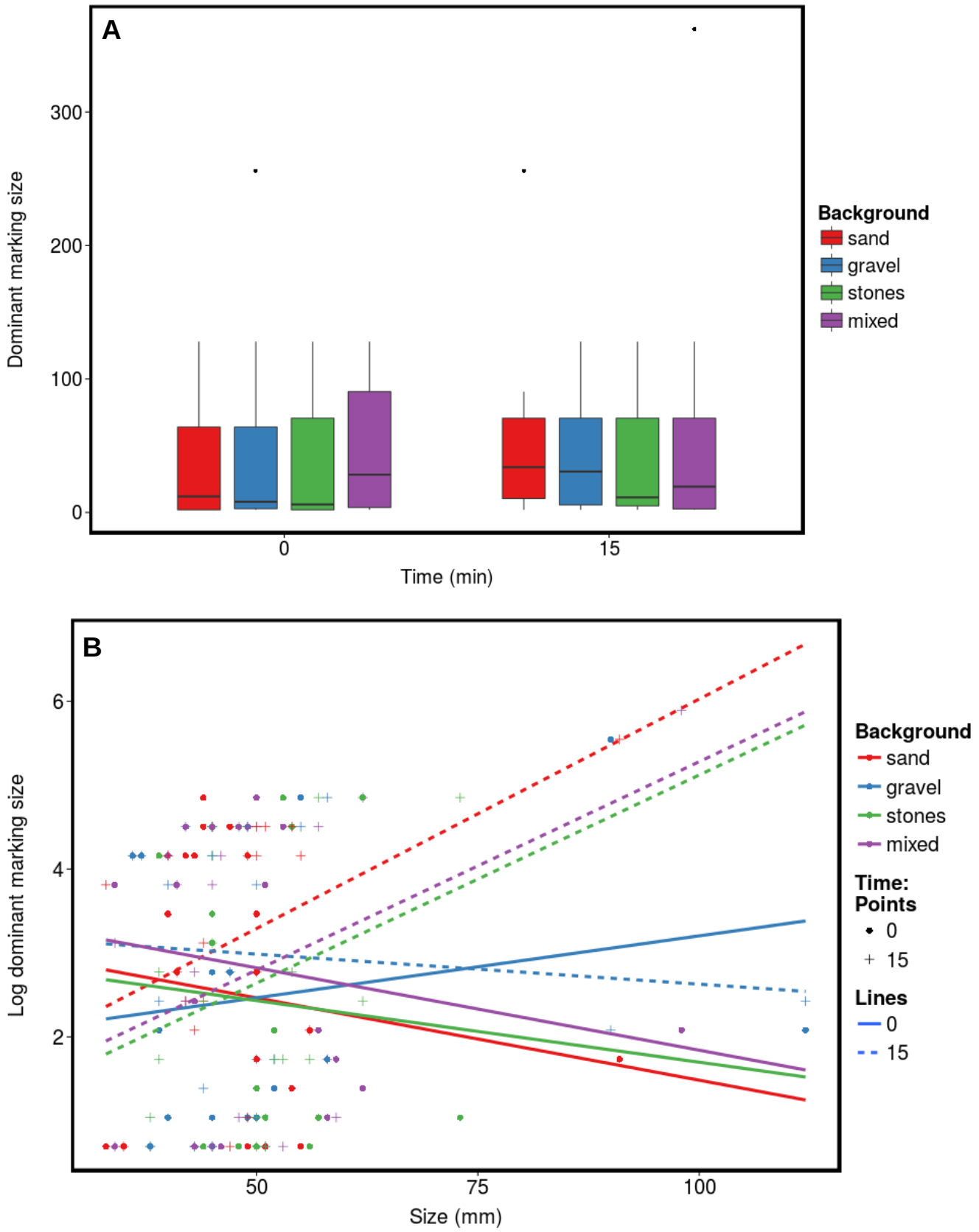

Figure 3.10: Change in dominant marking size over time for fish tested on the sand, gravel, stone, and mixed substrate backgrounds in experiment 2. Overall, there was little change in the most dominant marking size over time although fish greater than ~70 mm generally showed an increase in dominant marking size after 15 min (with the exception of those placed on the gravel background. (A) Dominant marking size of fish at 0, and 15 min. Graph shows medians plus inter-quartile range (IQR), whiskers are lowest and highest values that are within 1.5*IQR from the upper and lower quartiles, outliers are shown by dots. (B) Dominant marking size of fish at 0, and 15 min against fish size. Lines show general linear models for fish on the four backgrounds at each time point.

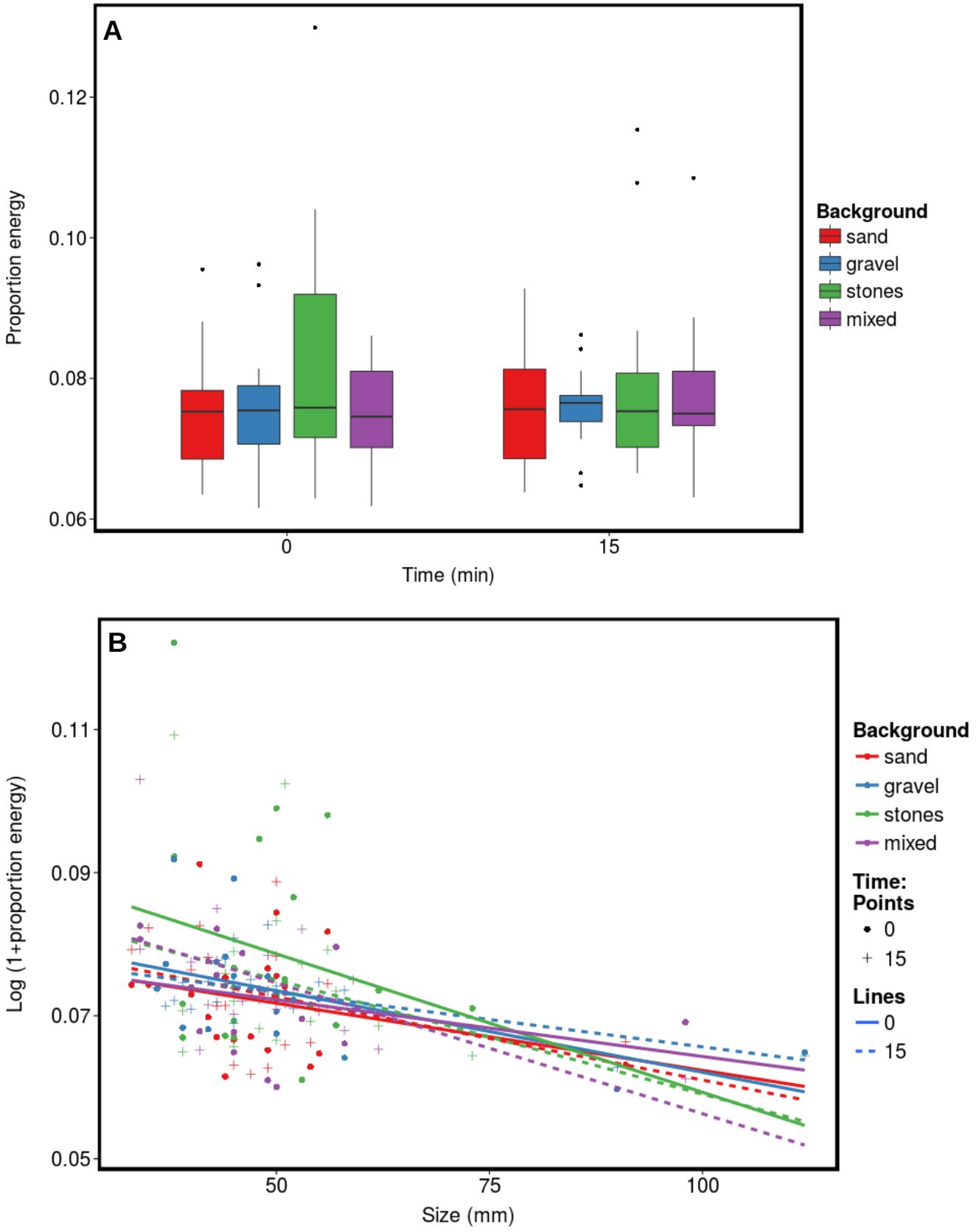

Figure 3.11: Change in pattern diversity, or the importance of the dominant marking size, over time for fish tested on the sand, gravel, stone, and mixed substrate backgrounds in experiment 2. There was no change in pattern diversity over time for fish on any of the backgrounds. There was however an overall increase in pattern diversity with increasing fish size. (A) Pattern diversity of fish at 0, and 15 min. Graph shows medians plus inter-quartile range (IQR), whiskers are lowest and highest values that are within 1.5*IQR from the upper and lower quartiles, outliers are shown by dots. (B) Pattern diversity of fish at 0, and 15 min against fish size. Lines show general linear models for fish on the four backgrounds at each time point.

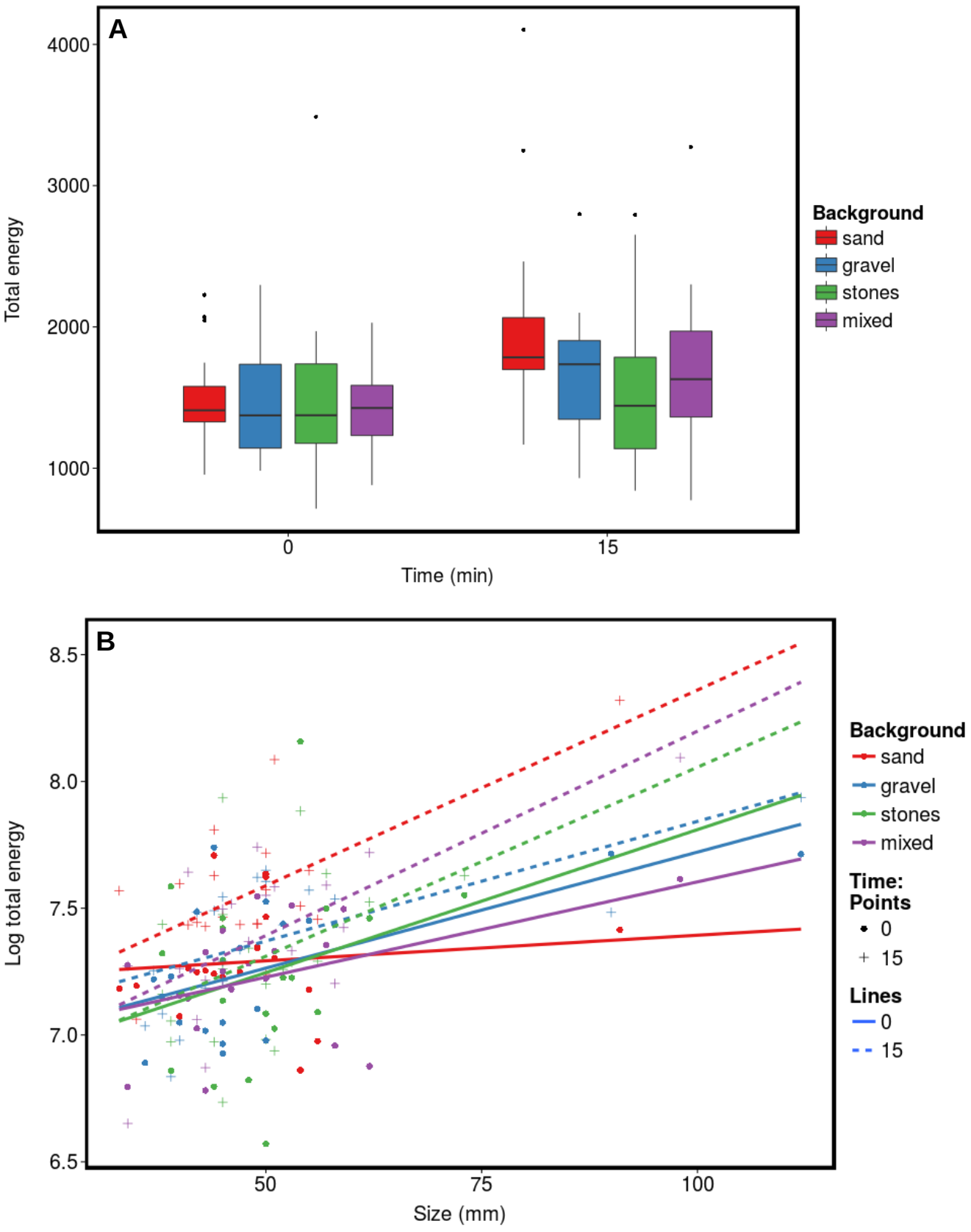

Figure 3.12: Change in pattern contrast over time for fish tested on the sand, gravel, stone, and mixed substrate backgrounds in experiment 2. There was an increase in pattern contrast after 15 min for fish placed on the sand background, and a small increase for fish placed on the gravel and mixed backgrounds. Fish greater than ~70 mm showed the greatest increase in pattern contrast. In general, larger fish tended to have more contrasting patterns. (A) Pattern contrast of fish at 0, and 15 min. Graph shows medians plus inter-quartile range (IQR), whiskers are lowest and highest values that are within 1.5*IQR from the upper and lower quartiles, outliers are shown by dots. (B) Pattern contrast of fish at 0, and 15 min against fish size. Lines show general linear models for fish on the four backgrounds at each time point.

Discussion

Here, I tested whether rock gobies can change their body pattern depending on the background on which they are placed. In experiment 1, fish were placed on a checkerboard background that had either 'small' (1 x 1 mm) or 'large' (5 x 5 mm) squares. Rock gobies were found to change their body pattern in response to both backgrounds, and both check sizes were found to elicit the same degree of pattern change. Body size was found to have a significant effect on pattern change, whereby larger fish displayed a greater change in pattern than smaller individuals. Fish were more camouflaged on the small checkerboard than on the large checkerboard even before being placed on the backgrounds. There was very little change in overall camouflage over time, although there was an improvement in camouflage as fish size increased. It is worth noting that the reason large fish appear rare in this study is because unlike smaller individuals that were able to shelter under stones where they were easily sampled the largest fish tended to hide in crevices within the rock at the edge of the rockpools. Sampling large individuals was therefore more difficult or at times impossible.

In experiment 2, fish were tested on grey scale images of different sized black and white substrates; sand (fine substrate size), gravel (medium substrate size), pebbles (large substrate size), and a mixture of all three. All four backgrounds elicited a change in body pattern; however there was a significant difference between backgrounds. The greatest change in pattern was observed in fish tested on the sand background, while the smallest change was observed in fish tested on the gravel and stones background. The degree of pattern change on the mixed background was somewhere in between that seen on the sand background and the gravel and stones backgrounds (when fish size was taken into account). All four backgrounds were grey-scale and matched in brightness, meaning that the fish were responding to differences in background pattern and spatial frequency and not overall achromatic or chromatic differences. As was the case in the first experiment, body size was found to have a significant effect on pattern change, although this was much weaker or non-existent in fish on the stones background. Even before being placed on the backgrounds, fish were most camouflaged on stones and least camouflaged on gravel. Camouflage on the sand and mixed background was intermediate between stones and gravel. The fish exhibited a limited improvement in camouflage after 15 min, which became more pronounced as the size of the fish increased. As would be expected, the difference in pattern between the fish and the

background was much greater on the checkerboards than the images of natural substrates, showing that camouflage was much better on more natural backgrounds.

Overall, in both experiments pattern change was very rapid with the majority of change occurring within the first minute of being placed on the experimental background. Rock gobies are therefore able to change pattern as rapidly as they can change colour and luminance (Stevens et al., 2014a). These results not only confirm that rock gobies can change their body pattern, but also demonstrate that they do so in response to changes in their background. Furthermore, to my knowledge this study is one of the first to investigate the role that specific background features, in this case substrate size, have in influencing pattern change for camouflage in fish other than flatfish.

In experiment 1 both checkerboard sizes were found to elicit the same degree of pattern change, suggesting that the check size was not important. Rock gobies may therefore differ from other marine species such as cuttlefish and flatfish in their response to different sized checkerboards. It is well established that the size of the squares on a checkerboard affects the corresponding pattern elicited by both cuttlefish (Barbosa et al., 2007; Chiao and Hanlon, 2001a; Chiao et al., 2007) and flatfish (Ramachandran et al., 1996). Chiao and Hanlon (2001a), and Ramachandran et al. (1996) tested the cuttlefish *Sepia pharaonis* and the flatfish *Bothus ocellatus* respectively on small versus large checkerboards very similar to those used in this study. Both *S. pharaonis* and *B. ocellatus* expressed a different pattern on the small checkerboard to that expressed on the large checkerboard. It is possible that the size difference between the two backgrounds used in this study was not large enough to result in a meaningful difference in pattern. However, although checks smaller than those on the small checkerboard were not tested, larger check sizes were tested in the preliminary experiment but were not found to elicit a noticeable change in pattern during observations.

Unlike cuttlefish, and to a lesser extend flatfish, which have a repertoire of different body patterns (Hanlon and Messenger, 1988; Kelman et al., 2006), it is likely that the rock goby has a more limited repertoire of patterns. This is supported in part by the descriptive statistics that were calculated in addition to PED. Overall there was little change in the predominant marking size, but there did appear to be a small increase in the relative importance of the most dominant pattern within fish tested on the small checkerboard (i.e. less marking diversity). This suggests that the marking size of the main component of the

fish's body pattern remains relatively constant between backgrounds, but the main component is expressed more on the small checkerboard than on the large checkerboard (though the difference in expression was not enough to cause a significant difference in PED between the two backgrounds).

In experiment 2 the change in pattern over time was significantly higher on the fine substrate (i.e. sand) compared to the other three backgrounds, indicating the fish respond most to high frequency background markings. The smallest change in body pattern was observed in fish tested on the medium (i.e. gravel) and large (i.e. stones) sized substrates suggesting lower frequency markings elicit a weaker pattern response than high frequency markings. Interestingly, when the fish were exposed to a background consisting of all three substrate sizes the resulting PED was similar to that elicited by the medium and large substrates, though both the medium and mean were higher on the mixed background. This infers that the fish take into account information about all spatial scales when changing body pattern. If high frequency markings were more important than other sizes then one would expect the PED on the mixed background to be the same or more similar to that on the sand background. Like the mixed background, real rockpools consist of many different substrates of different sizes. It may therefore be adaptive for fish to take into account, and respond to, all spatial scales thus allowing them to elicit the most appropriate change in pattern for their background.

As was the case on the checkerboards, overall there was no change in the dominant marking size nor was there any change in the importance of the dominant marking size. However, fish on the sand background (high spatial frequency) elicited a more contrasting pattern than fish on the other three substrates. Rock gobies therefore differ from cuttlefish which have been found to elicit more contrasting patterns when exposed to medium or low spatial frequency backgrounds and are less contrasting on high spatial frequency backgrounds (Chiao et al., 2009). Based on this it can be speculated that rock gobies are likely to utilise a different camouflage strategy to that employed by cuttlefish. It is also possible that rock gobies are responding to different features in the background from those used by cuttlefish.

In both experiments there was a positive correlation between fish size and the amount of pattern change, whereby larger fish showed a greater change in pattern. The type of pattern expressed by cuttlefish depends on the size of the individual substrate components relative to

the size of the cuttlefish (Barbosa et al., 2004, 2007; Chiao and Hanlon, 2001a). For instance, when placed on a black and white checkerboard, a checker size approximately 30% to 120% of the area of the 'white square' component (a rectangular area located in the centre of the dorsal mantle of cuttlefish) elicited a so-called disruptive pattern, regardless of the actual size of the cuttlefish (Barbosa et al., 2007; Chiao and Hanlon, 2001a). On checker sizes smaller or larger than this, cuttlefish generally show uniform or mottle body patterns (Barbosa et al., 2004, 2007; Chiao and Hanlon, 2001a). To determine if the size of the substrate components relative to the size of the fish is important in rock gobies I calculated the relative size of the sand, gravel, and stone substrates as a percentage of the approximate area (excluding the gills, eyes, and pectoral and caudal fins) of each fish. Sand was <1% of the area of the fish, gravel ranged from ~4% to ~40%, while stones ranged from ~90% to ~600%. There was a negative correlation between the amount of pattern change and the relative size of the substrates (i.e. smaller fish change pattern less), until the substrate components were above 100% of the area of the fish at which point there was no correlation between relative substrate size and the amount of pattern change. However, based on the fact that both the gravel and stone backgrounds elicited the same PED between 0 and 15 min, the positive correlation between the amount of pattern change and fish size is likely to be the result of absolute, rather than relative, fish size, providing the substrate is smaller than the total area of the fish. Nevertheless, an experiment whereby fish of different sizes are placed on a background composed of components that are identical in size relative to the size of the fish is needed to determine whether it is the absolute or relative fish size that is most important.

As discussed above, the change in pattern over time for fish tested on the checkerboards was greater than the change in pattern observed in fish tested on the backgrounds in experiment 2. Camouflage was much poorer on the checkerboard backgrounds compared to the images of sand, gravel, stones and mixed substrates and so this may have been why they elicited a greater change in body pattern. However, this is unlikely to be the whole story because in experiment 2 fish were least camouflaged on the gravel background yet gravel did not elicit the greatest change in pattern. An alternative explanation could be differences in pattern contrast. The backgrounds used in the first experiment were more contrasting, as least from a human perspective, than those used in the second experiment. This is because it used black and white squares while the photos used in experiment 2 were grey-scaled so the white substrate was light grey (and not paper white) while the black substrate was dark grey rather than black. In cuttlefish it has been shown that

the type of body pattern expressed is highly dependent on background contrast (Barbosa et al., 2008b; Chiao and Hanlon, 2001a; Chiao et al., 2007), whereby the greater the perceived contrast of the background, the bolder the pattern expressed by the cuttlefish (Chiao and Hanlon, 2001a). Moreover, when placed on a low contrast checkerboard cuttlefish are known to express a uniform like pattern irrespective of check size (Barbosa et al., 2008b). It may therefore be that pattern change in rock gobies is also greater on more contrasting backgrounds.

It is also possible that the amount of edge information in the background may influence pattern change. Visual edges are often perceived as abrupt changes in intensity commonly associated with object borders such as those between the black and white squares on a checkerboard (Stevens & Cuthill 2006). By this definition, the checkerboard backgrounds tested in the first experiment have stronger edge information than the images of different substrates used in the second experiment. Edge information has been found to be important in influencing pattern change in cuttlefish whereby backgrounds containing strong edge information elicited boulder, more contrasting body markings compared to backgrounds with reduced or no edge information (Chiao & Hanlon 2001; Chiao et al. 2004; Kelman et al. 2007; Zylinski et al. 2009). To my knowledge no literature exists on the importance of edge information or substrate contrast in influencing changes in body pattern in fish and this is an area for future research.

Above it was suggested that rock gobies are likely to have a limited repertoire of one or two patterns which they express to varying degrees. This is similar to many flatfish, which match their background using a limited number of pre-set internal patterns (Lanzing 1977; Tyrie et al. 2015), though there can still be considerable variation between individuals, and even within the same individual, on the same background (Healey, 1999). For instance, the tropical flatfish *Bothus ocellatus* is thought to have three basic patterns (Ramachandran et al., 1996), southern flounder (*Paralichthys lethostigma*) and winter flounder (*Pseudopleuronectes americanus*) have at least one pattern, and plaice (*Pleuronecte platessa*) have two (Kelman et al., 2006). In addition Nassau groupers (*Epinephelus striatus*) have also been shown to change between three basic body patterns within a few seconds (Watson et al., 2014).

Observations made during the experiments and in the field suggest that rock gobies altered the expression of one of two different pattern types. The two pattern types, here referred to as ‘striped’ and ‘black square’, are shown in Figure 3.13. It is not known whether a given individual is able to elicit both pattern types, as this was not observed in my study. It is, however, unlikely that these two pattern types are mutually exclusive and there are many similarities between them (e.g. Figure 3.13c and e). It should also be noted that the ‘striped’ pattern type was not observed in fish greater than 60 mm in length. The ‘black square’ pattern type was observed in fish of all sizes, but was most vivid and contrasting in larger individuals. While it is possible that these two pattern types result from sexual dimorphism, this has not been reported in any of the studies which investigated the life history of this species (Azevedo and Simas, 2000; Dunne, 1978; Hajji, 2012; Miller, 1961). While these markings could play a role in some form of signalling the fact that the fish changed their pattern in response to different backgrounds suggests that they are, at least in part, important for camouflage. Furthermore, because fresh sea water was used for each fish, and there was no movement of water between compartments, any pattern change in response to potential chemical cues from conspecifics was eliminated.

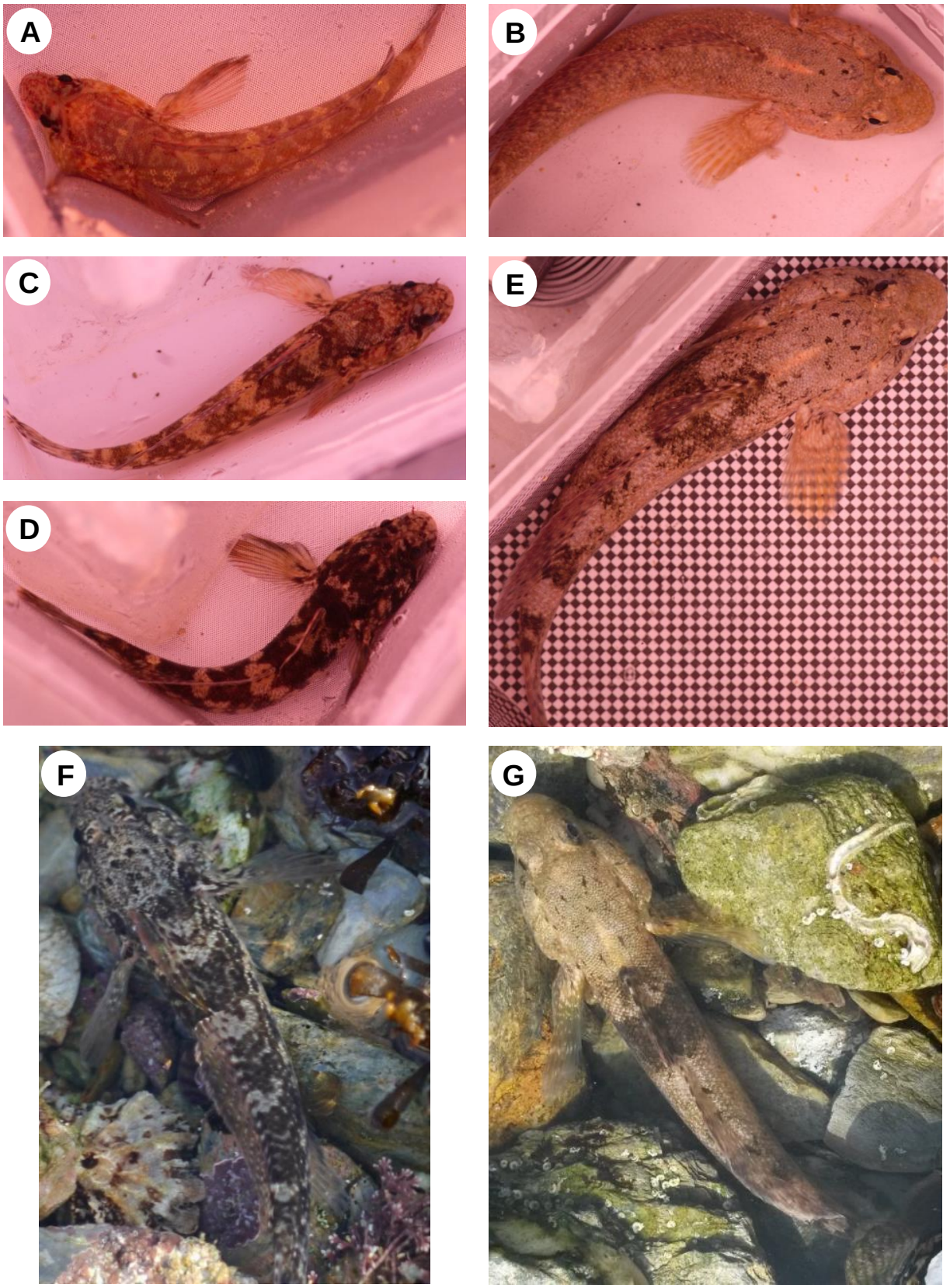

Figure 3.13: The two basic pattern types, here referred to as 'striped' (left) and 'black square' (right), identified in rock gobies on Gyllyngvase beach, Falmouth. (A) Striped pattern not expressed, (B) black square pattern not expressed, (C) striped pattern partially expressed, (D) striped pattern fully expressed, (E) black square pattern partially expressed, (F) striped pattern fully expressed while observing the rock goby in a rockpool, and (G) black square pattern fully expressed while observing the rock goby in a rockpool. Photo credit: Sam Smithers (A-E) and Alice Lown (F-G).

Due to the nature of rocky shores as heterogeneous environments, closely matching the background with fixed patterns is challenging and depends on the composition of the habitat patch. Instead, the patterning of rock gobies may have evolved as a compromise to camouflage on multiple backgrounds rather than to specialise on one background type (Houston et al., 2007; Merilaita et al., 1999; Stevens, 2007). However, compared to the background matching ability of other pattern changing animals, rock gobies showed a very limited improvement in background match in almost all but the largest individuals, despite showing a large change in body pattern on all backgrounds. This suggests that background matching might not be the primary function of the body markings of rock gobies. For instance, both the striped and black square pattern types crossover the edge of the body which is characteristic of disruptive coloration (Cuthill et al., 2005). Fast visual detection of animals in natural scenes has been shown to depend heavily on information regarding visual edge and body outline while chromatic information is of little importance (Delorme et al., 2000; Elder and Velisavljević, 2009; Fei-Fei et al., 2005). Disruptive coloration may therefore provide a survival advantage for rockpool fish and potentially even outweigh the benefits of background matching alone. Furthermore, it has been shown that disruptive camouflage can be an effective anti-predator strategy even if the overall combination of markings do not match the background entirely (Schaefer and Stobbe, 2006; Stevens and Cuthill, 2006).

Another potential function of the body patterns might be motion dazzle (Stevens et al., 2008). Camouflage through motion dazzle would be particularly beneficial in rockpools as fish are often clearly visible through the surface of the water (sometimes from several metres away) when swimming. As such the expression of dazzle markings may be expected to provide a survival advantage, in particular against avian predators that could easily see a moving fish through the water. It is however important to consider that uniform coloration has also been shown to provide camouflage during motion (Stevens et al., 2008), and as such motion dazzle alone is unlikely to be the primary function of the body pattern of rock gobies. Disruptive coloration, or possibly a combination of disruption and motion dazzle, is therefore a more likely explanation for the function of these body patterns.

The backgrounds used in experiment 2 were designed to be representative of natural substrates at different spatial scales while controlling for differences in chromatic and achromatic information that would have existed in the original substrates. The use of photos also demonstrates that the fish were responding to visual, rather than textural, cues. The same

has also been found in cuttlefish (Chiao et al. 2005; Kelman et al. 2008). The limitation is that I cannot be sure that the fish would respond in the same way to real substrates. For instance, plaice have been shown to change pattern almost instantly when moved from fine to coarse gravel of the same hue, but respond very differently when moved between artificial backgrounds (Healey, 1999). Even cuttlefish have been found to show a stronger pattern change on real gravel than a 2D image of the gravel, though there was no difference between cuttlefish which were directly on the gravel and those viewing it through Perspex, indicating that they are indeed using visual cues (Kelman et al., 2008). None of the backgrounds used in this study elicited the full expression of the 'black square' in any of the individuals tested. The full expression of the 'black square' was only seen while observing the fish within the rockpools (Figure 3.13g), suggesting that cues not present in the experimental backgrounds are also important. Alternatively, if these markings are involved in motion dazzle then they may only be expressed fully during motion.

This study has shown that rock gobies are capable of rapidly changing their body pattern in response to changes in their visual background. The ability to change body pattern could potentially be wide spread among rockpool fish, as well as other littoral species. Future research should aim to explore the extent to which the markings of rock gobies, when expressed, function as disruptive coloration (and potentially motion dazzle). It would also be interesting to investigate if rapid pattern change is indeed widespread amongst other intertidal species of fish.

Chapter 4: General Discussion

Photo credit: Alice Lown

The work in this thesis explores the extent to which rock gobies are capable of changing their colour, luminance, and pattern to match different backgrounds on which they are placed. In chapter 2 I found that rock gobies become redder and more saturated when placed on a background that resembles the colour of sand than when they are placed on a background resembling the colour of green algae covered rock. This result is in accordance with previous work that found gobies turn redder when placed on a red background (Stevens et al., 2014a). More noteworthy was the fact that rock gobies were more camouflaged on the sand coloured background than the rock background. I put forward the suggestion that this could be the result of a higher selection pressure to match the colour of sand compared to other colours such as blue or green. This is because sand can form large homogenous habitats in areas where it is a predominate substrate. Although rock gobies tend to occur in rocky shores rather than sandy shores, often the two are mixed meaning the action of waves and currents may force fish onto other habitat types such as sandy patches. Since sand dominated habitats offer relatively few places for fish to hide (other than burial) one would expect an increased selection pressure on fish to match the colour of their background to avoid predation. In contrast, rockpools are extremely heterogeneous and consist of many substrate types of different colours, and so pressure to match any single colour, such as the greenish grey colour of the rock background used in my experiment, may be smaller. Moreover rockpools provide fish with numerous places to hide from predators such as under stones or within rock crevices. Predation risk also tends to be lower in complex heterogeneous habitats such as rockpools as complex habitats tend to provide better protection from predators by impeding prey detection (Bond and Kamil, 2006; Dimitrova and Merilaita, 2012; Stoner and Titgen, 2003). It should also be noted that red/orange colours are far more common in rockpool environments than green (and blue which was tested in Stevens et al. (2014a)) and the ability to become redder would enable rock gobies to match the colour of other backgrounds such as red algae as well as brown rocks.

The ability to match the sand colour more easily could also be linked to the different types of chromatophores that control colour change. For instance xantophores contain the yellow pigment pteridine and erythrophores contain red carotenoids (Sköld et al., 2013). Together these two types of chromatophores allow fish to match yellow and reddish backgrounds such as sand. In contrast the majority of fishes do not have pigment cells with a blue-green hue (Sköld et al., 2013). Green or blue can therefore only be achieved by a more complex arrangement whereby xanthophores and melanphores are overlayed by iridophores

or leucophores (Sköld et al., 2013). The gobies could therefore only match the green hue of the rock background if they possessed such an arrangement of chromatophores. To my knowledge it is not known whether this is the case or not.

In chapter 2 I also found that rock gobies display a strong behavioural preference for dark backgrounds over light ones irrespective of their previous background. Similarly acclimatisation background had no effect on preference when fish were given a choice between the sand and rock backgrounds. However, unlike on black and white when all fish showed a strong overall preference for black, there was no significant overall preference for either the sand or the rock background. In chapter 3, I found that rock gobies change their body pattern within one minute when placed on a checkerboard with squares measuring either 1 x 1 mm or 5 x 5 mm. Rock gobies were also found to change pattern when placed on grey-scale images of different sized substrates designed to represent backgrounds of different spatial scales with the high spatial frequency background eliciting the greatest change in pattern.

The variety of colours in rockpools may mean predators are more reliant on other cues such as body outline and shape instead of colour to locate prey. Indeed, for primates at least, there is good evidence that fast visual detection of animals in natural scenes does not depend upon colour, rather information on visual edge is far more important (Delorme et al., 2000; Elder and Velisavljević, 2009; Fei-Fei et al., 2005). If so disruptive markings may provide a survival advantage supporting the speculation from the previous chapters that body pattern may be more important for camouflage than the overall colour and lightness match to the background. Both the striped and black square pattern types have characteristics of disruptive coloration in that they overlap the edge of the body when viewed directly from above or the side (Cuthill et al., 2005). Thus the markings may help to create the illusion of false edges in order to break up the outline of the fish so as to inhibit detection (Cuthill et al., 2005; Stevens and Cuthill, 2006). Furthermore, it has been suggested that disruptive coloration can provide an effective anti-predator defence without being background matching (Schaefer and Stobbe, 2006), perhaps by hindering recognition rather than impeding initial detection (Webster et al., 2013). The implication of this is that rockpool fish could potentially be camouflaged on a variety of backgrounds even if their background matching ability differs between substrates. This suggestion should, however, be taken with some caution as other research has found that while disruptive patterns which do not match the background provide some protection, they

are not as effective as disruptive patterns that do match the overall appearance of the background (Stevens et al., 2006). Patterns which are characteristic of disruptive coloration have been identified in other rapid colour changing species of fish such as the Nassau grouper (*Epinephelus striatus*) (Watson et al., 2014) and the slender filefish (*Monacanthus tuckeri*) (Allen et al., 2015). Disruptive coloration is also apparently common among fishes with fixed patterns such as anglerfish (e.g. *Antennarius commerson*) and damselfish (e.g. *Dascyllus aruanus* and *D. reticulatus*) (Marshall and Johnsen, 2011).

Motion dazzle was also suggested in chapter 3 as a possible explanation for the function of the patterns expressed by rock gobies on different backgrounds. While patterns such as stripes have been found to provide camouflage in the form of motion dazzle, studies have shown that uniform coloration also provides camouflage during motion (Stevens et al., 2008). Motion dazzle alone is therefore unlikely to be the primary function of the body pattern of rock gobies and other rockpool fish thus disruptive coloration, or possibly a combination of disruption and motion dazzle, is more likely.

Future research

Perhaps the most exciting area for future research is following on from the findings of chapter 3. Firstly the suggestion that rock gobies may be using disruptive coloration needs testing directly to determine if indeed this is the case. The suggestion that the fish may also be using motion dazzle in addition to disruptive coloration also needs testing empirically. Although there was little support for it in this study, it is also possible that the patterns may be involved with background matching. This could be tested by in situ field experiments in which fish are photographed within rockpools allowing the markings of each individual to be compared directly against the colour and pattern of its natural background. Unfortunately such a study would be very difficult using the techniques used throughout this thesis. However a study using a form of focal animal sampling in a similar way to Allen et al. (2015), whereby one individual is recorded for a set period of time, or for as long as feasible, might be a viable alternative.

Beyond rock gobies colour change has been shown in at least four other species of goby (Fries, 1942; Gaisner, 2005; Goda and Fujii, 1996; Sköld et al., 2008), and it is likely that colour change is widespread within this group, though the function may differ between

species. It would be interesting to determine how the colour and pattern changing ability of the rock goby compares to other species, particularly those from the tropics that will have evolved under different environmental conditions. Colour change for camouflage has also been documented in a number of other rockpool species including the shore crab (*Carcinus maenas*) (Stevens et al., 2014b), chameleon prawn (*Hippolyte varians*) (Keeble and Gamble, 1899), and more recently the shanny, or common blenny, (*Lipophrys pholis*) (Hesse, Smithers and Stevens, unpublished data). There is therefore scope for comparative studies using other species of rockpool fish.

Despite the large body of research on colour and pattern change in fish we still have a poor understanding of the cues the fish are using to choose the optimum colour pattern to express when exposed to a new background. For instance, the size, contrast, and number of light coloured background elements are important in influencing the type of pattern expressed by cuttlefish (Barbosa et al., 2008b; Chiao and Hanlon, 2001a; Chiao et al., 2007). However little is known about the importance of these cues in fish, thus generating a host of unanswered questions making this an exciting area for future research. Furthermore, gobies make an ideal model system for answering these questions since gobiid gobies constitute the largest family of marine fish in the world with over 1950 species (Helfman et al., 2009).

This study used artificially generated backgrounds designed to be representative of those found in rockpools, while allowing me to manipulate a single factor and keeping all other cues the same. While this did allow me to determine the response of rock gobies to specific cues, this approach nevertheless has its limitations. This is due to the fact that in nature fish are likely to be responding to multiple cues in their environment and no single background feature is likely to explain all variation. For instance, in chapter 3 I manipulated the spatial frequency of the backgrounds while removing chromatic and textural information, both of which may be important in influencing pattern change. While studies such as this allow us to determine which factors are important, we cannot ignore the fact that the fish are tested under conditions that do not exist in nature, thus generalisation to the real world is limited. Future research should therefore look to extend this study using natural substrates in addition to the more controlled artificial backgrounds. This has been done well within research on cuttlefish, whereby studies utilising artificial backgrounds such as the checkerboard (e.g. Chiao and Hanlon, 2001a; Barbosa et al., 2007) are complemented, and

more crucially supported, by studies using natural backgrounds (e.g. Mäthger et al., 2007, 2008).

Closing words

Small rockpool fish achieve crypsis through a combination of rapid physiological colour change and behavioural background matching. Behavioural background matching may be of particular importance in areas where the fish's ability to match a particular substrate is more limited, such as when exposed to light coloured backgrounds. Rock gobies are able to adapt to new backgrounds by changing not only the colour and luminance of their skin, but also their body pattern. The degree to which fish change body pattern is affected by the size of the substrate making up the background. The next step will be to perform experiments to determine if the striped and black square pattern types function as disruptive coloration.

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