Anti-predator Adaptations in Aquatic Environments

This thesis investigates how protective coloration can be used, either as such or together with behavioural adaptations, to manipulate predator behaviour and decrease predation risk. The results demonstrate that protective coloration provides numerous distinctive ways for aquatic prey to escape predation. Thus, visual perception and behaviour of fish are important factors shaping the appearance and behaviours of aquatic prey.
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“I reject your reality and substitute my own!”

ABSTRACT
Predation is an important selective force that has led to the evolution of a variety of fascinating anti-predator adaptations, such as many types of protective coloration and prey behaviours. Because the evolution of life has begun in the aquatic environment and many anti-predator adaptations are found already in relative primitive taxa, it is likely that many of these adaptations evolved initially in the aquatic environment. Yet, there has been surprisingly little research on the mechanisms and function of anti-predator adaptations in aquatic systems. To understand the function of anti-predator adaptations and natural selection imposed on prey appearance and behaviour, I have investigated how protective coloration can be used, either as such or together with behavioural adaptations, to manipulate predator behaviour and decrease predation risk. To this end I conducted a series of behaviour ecological laboratory experiments in which I manipulated the visual appearance of artificial backgrounds and prey items.

In paper I of this thesis, I investigated background choice as an anti-predator strategy, by observing the habitat choice of the least killifish (*Heterandria formosa*) between pairs of artificial backgrounds, both in the presence and absence of predation threat. It has been suggested that prey could decrease their risk of being detected by predators either by preferring backgrounds into which they blend or by preferring visually complex backgrounds. The least killifish preferred a background that matched their patterning to a background that mismatched it, showing that they are able to respond to cues of visual similarity between their colour pattern and the surrounding environment. Interestingly however, in female least killifish visual complexity of the background was a more important cue for habitat safety and may override or act together with background matching when searching for a safe habitat. It is possible that in females, preference for visually complex backgrounds is associated with lower opportunity costs than preference for matching backgrounds would be. Generally, the
least killifish showed stronger preference while under predation threat, indicating that their background choice behaviour is an anti-predator adaptation.

Many aquatic prey species have eyespots, which are colour patterns that consist of roughly concentric rings and have received their name because they for humans often resemble the vertebrate eye. I investigated the anti-predator function of eyespots against predation by fish in papers II, III and IV. Some eyespots have been suggested to benefit prey by diverting the strikes of predators away from vital parts of the prey body or towards a direction that facilitates prey escape. Although proposed over a century ago, the divertive effect of eyespots has proven to be difficult to show experimentally. In papers II and III, I tested for divertive effect of eyespots towards attacking fish by presenting artificial prey with eyespots to laboratory reared three-spined sticklebacks (Gasterosteus aculeatus). I found that eyespots strongly influenced the behaviour of attacking sticklebacks and effectively drew their strikes towards the eyespots. To further investigate this divertive effect and whether the specific shape of eyespots is important for it, I tested in paper III the response of fish also to other markings than eyespots. I found that eyespots were generally more effective in diverting the first strikes of attacking fish compared to other prey markings. My findings suggest that the common occurrence of eyespots in aquatic prey species can at least partly be explained by the divertive effect of the eyespot shape, possibly together with the relative simple developmental mechanisms underlying circular colour patterns.

An eyebar is a stripe that runs through the eye, and this pattern has been suggested to obscure the real eyes of the prey by visually blending parts of the eyes and head of the prey and by creating false edges. In paper III, I show that an eyebar effectively disrupts an eyeliike shape. This suggests that eyebars provide an effective way to conceal the eyes and consequently obstruct detection and recognition of prey. This experiment also demonstrates that through concealment of the eyes, eyebars could be used to enhance
the divertive effect of eyespots, which can explain the common occurrence of eyebars in many species of fish that have eyespots.

Larger eyespots have been shown to intimidate some terrestrial predators, such as passerine birds, either because they resemble the eyes of the predator’s own enemy or because highly salient features may have an intimidating effect. In papers II and IV, I investigated whether the occurrence of eyespots in some aquatic prey could be explained by their intimidating effect predatory fish. In paper IV, I also investigated the reason for the intimidating effect of eyelike prey marks. In paper II, I found no clear intimidating effect of eyespots, whereas in paper IV, using a different approach, I found that sticklebacks hesitated to attack towards eyelike but not towards non-eyelike marks. Importantly, paper IV therefore presents the first rigorous evidence for the idea that eye mimicry, and not merely conspicuousness, underlies the intimidating effect. It also showed that the hesitation shown by fish towards eyelike marks is partly an innate response that is reinforced by encounters with predators.

Collectively, this thesis shows that prey colour pattern and the visual appearance of the habitat influence the behaviour of fish. The results demonstrate that protective coloration provides numerous distinctive ways for aquatic prey to escape predation. Thus, visual perception and behaviour of fish are important factors shaping the appearance and behaviours of aquatic prey.

**SAMMANFATTNING**

Predation är ett viktigt selektionstryck som har lett till evolutionen av en mängd olika fascinerande antipredator anpassningar såsom många typer av skyddsfärgteckningar och beteenden hos bytesdjur. Eftersom evolutionen av livsformer har sitt ursprung i den akvatiska miljön och många anpassningar mot predation upptäckts redan hos relativt primitiva djurtaxa, är det även troligt att många utav dessa anpassningar ursprungligen evolverat i akvatiska miljöer. Ändå har förvånansvärt lite forskning studerat mekanismerna och funktionerna av anpassningar mot predation i akvatiska miljöer. För att förstå funktionen av anpassningar mot
predation och den naturliga selektionen på bytesdjurs utseende och beteende, har jag undersökt hur skyddsfärgteckningar kan användas, antingen som sådana eller tillsammans med beteendemässiga anpassningar, för att manipulera predatorers beteende och på så vis minska predationsrisken. Med detta som målsättning utförde jag en serie av beteendeekologiska laboratorieexperiment där jag manipulerade det visuella utseendet hos artificiella byte och bakgrunder.

I studie I undersökte jag bytesjurs användning av bakgrunder som anpassning mot predation genom hur dvärgtandkarpar (Heterandria formosa) valde habitat mellan par av artificiella bakgrunder, både med och utan en predator närvarande. Det har tidigare föreslagits att bytesjur kan minska sin upptäcktsrisk antingen genom att välja bakgrunder där deras färgteckning smälter in eller genom att välja bakgrunder som är visuellt komplexa. Dvärgtandkarparna föredrog bakgrunden med mönster som likade fiskarnas eget mönster framom en bakgrund med mönster som inte liknade det, vilket visar att omgivande miljöns visuella likhet med deras eget mönster påverkar tandkarpars habitatsval. En intressant upptäckt hos dvärgtandkarphonor var att visuell komplexitet hos bakgrunden var en viktigare signal för habitatstrygghet, och kan därför övertrumfa eller verka tillsammans med visuell likhet med bakgrunden i sökandet efter ett tryggt habitat. Det är möjligt att preferens för visuell komplexitet hos dvärgtandkarphonor kan leda till lägre kostnader i form av förlorade tillfällen att delta i andra fitnesshöjande aktiviteter än vad preferens för visuell likhet med bakgrunden skulle göra. Generellt så visade dvärgtandkarparna starkare preferens för endera bakgrunden under predationshot, vilket tyder på att beteendet är en anpassning mot predation.

Många akvatiska bytesdjur har ögonfläckar, vilka är färgmönster som består av ungefärligt koncentriska ringar och som har fått sitt namn för att de, åtminstone för människor, liknar vertebratögon. Jag undersökte funktionen av ögonfläckar mot predation av fisk i studierna II, III och IV. Somliga ögonfläckar har föreslagits vara fördelaktiga för bytesjur genom att rikta predatorers hugg bort

Ett ögonstreck är en rand som löper igenom ögat, och detta mönster har föreslagits dölja bytesdjurets egna ögon genom att visuellt smälta samman delar av ögat och huvudet på bytesdjuret, samt genom att skapa falska kanter. I studie III visar jag att ett ögonstreck effektivt löser upp en ögonlik form. Detta tyder på att ögonstreck är ett effektivt sätt att dölja ögonen på, och på så vis hindra upptäckt och igenkännande av bytesdjur. Detta experiment visar även att genom att dölja de riktiga ögonen kan ögonstreck användas för att förstärka den riktande effekten av ögonfläckar, vilket kan förklara varför många fiskar som har ögonstreck även har ögonfläckar.

Stora ögonfläckar har visat sig fungera skrämmande mot vissa terrestriska predatorer som tättingar, aningen för att de liknar ögonen hos predatorns egen predator, eller för att mycket ögonfallande mönster generellt kan ha en skrämmande effekt. I studie II och IV undersökte jag huruvida förekomsten av ögonfläckar hos åtskilliga akvatiska bytesdjur kan förklaras av deras skrämmande effekt mot rovfiskar. I studie IV undersökte jag även den bakomliggande
orsaken till den skrämmande effekten av ögonlika mönster. I studie II fann jag ingen tydlig skrämmande effekt av ögonfläckar, medan det i studie IV där jag använde en annan metod, visade sig att spiggarna tvekade att anfalla byten med ögonlika mönster men inte att anfalla byten med icke-ögonlika mönster. Resultaten i studie IV ger därför det första rigorösa stödet för idén att det är ögonlikhet, och inte enbart iögonfallande, som ligger till grund för den skrämmande effekten. Studien visar även att den tvekan som fiskarna visade mot de ögonlika mönstren är delvis en medfödd respons som också förstärks utav erfarenhet av predatorer.

Sammandraget visar denna avhandling att mönster hos bytesdjur och utseendet hos habitatet påverkar fiskars beteende. Resultaten demonstrerar att skyddsfärgteckning ger många, distinkta möjligheter för akvatiska bytesdjur att undkomma predation. Därmed är visuell perception och beteende hos fisk viktiga faktorer i utformandet av utseende och beteende hos akvatiska bytesdjur.
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This thesis is based on the following papers, which are referred to in the text by their respective Roman numerals:


III  Kjernsmo, K. Grönholm, M. & Merilaita, S. Eyespots, eyebars and diversion of attacks by fish. *Manuscript*

IV  Kjernsmo, K. & Merilaita, S. Intimidating eye-mimicry and eyespots: eyelike prey marks cause hesitation in attacking fish. *Manuscript*

© Royal Society Publishing (*papers I and II*)
1. INTRODUCTION

1.1 Anti-predator adaptations

Predation is omnipresent, and the most susceptible prey individuals are killed even before they have reproduced. Therefore, predation is a strong selective force in nature. Predator-mediated selection favours those individuals whose traits allow them to postpone death until they have managed to reproduce at least once (Alcock 2005). This has led to the evolution of a large variety of anti-predator adaptations in prey and also to the evolution of counter-adaptations in predators (e.g. Darwin 1859). Anti-predator adaptations can be morphological (such as exoskeletons, spines and various types of protective prey colorations) and behavioural (such as avoiding dangerous areas, group formation or hiding in safe refuges) (e.g. Poulton 1890; Cott 1940; Ruxton et al. 2004; Nonacs & Blumstein 2010). Obviously, selection pressures other than predation also can have an important impact in shaping the appearance and behaviour of animals (e.g. sexually selected traits, naturally selected traits involved in intraspecific communication, traits that are involved in other fitness enhancing processes (thermoregulation)), but these are outside the scope of this thesis.

In this thesis I have focused on anti-predator adaptations in aquatic environments. This because even though most anti-predator defences have evolved in the aquatic milieu, surprisingly little has been studied about the adaptive value and functions of anti-predator defences in aquatic systems. One of my main aims has been to investigate predator-mediated selection imposed on or by fish. More specifically, I have focused on protective colouration and related behavioural adaptations, mainly habitat choice. All these anti-predator adaptations target the visual sensory modality of predators. Despite my use of aquatic organisms, many of my results have a broader importance due to the generality of the evolutionary question that I have addressed.

1.2 Protective coloration and related anti-predator behaviours

Protective coloration has intrigued naturalists and biologists already for a long time, and the potential adaptive value of camouflage was discussed by Charles Darwin’s grandfather Erasmus Darwin, who wrote that:
The colours of many animals seem adapted to the purpose of concealing themselves, either to avoid danger, or to spring upon their prey.”

(Darwin 1794)

There are many forms of protective coloration in animals, and these are commonly divided into two major categories: 1) coloration that prevents detection or recognition (i.e. various types of camouflage) and 2) coloration that decreases for a detected prey the risk of an attack or the harm it may cause (i.e. anti-predator signals, such as warning colouration, mimicry and eyespots) (Poulton 1890; Thayer 1909; Cott 1940; Ruxton et al. 2004). These two categories of protective coloration are also often associated with some behavioural adaptations. For example, to increase the efficacy of camouflage a prey may decrease its mobility during the times it has an elevated risk of encountering a predator (Ruxton et al. 2004). By studying protective coloration we can gain important information about why animals look and behave the way they do, and also increase our understanding of questions regarding predator psychology.

In this thesis, I have studied how prey can use colouration either alone or in combination with a behavioural adaptation to manipulate the behaviour of predators in a way that would either reduce the probability of being detected, attacked or to reduce the likelihood of an attack to be successful. I have primarily focussed on three types of protective colouration: crypsis, eyespots, and eyebars and will therefore confine myself to these three and address them separately below.

1.2.1 Crypsis (paper I)

The term crypsis includes traits that decrease the risk of becoming detected when potentially perceivable to an observer (Stevens & Merilaita 2009a). Crypsis comprises both morphological features such as background matching coloration, (colour patterns which generally resemble the colour and lightness of one or several background types, Endler 1978; Merilaita 1999; Ruxton 2004; Merilaita & Stevens 2011), and behavioural traits that enhance crypsis (Stevens & Merilaita 2009a). In this thesis, I have studied how animals can use behaviour to enhance their crypsis. Several experimental studies have shown that background matching (i.e. the similarity in appearance between a prey and its visual
background) effectively reduces predation risk (e.g. predation by fishes: Feltmate & Williams 1989; Merilaita 2001; Johnsson & Källman-Eriksson 2008; and birds: Kettlewell 1955; Cooper 1994; Merilaita & Dimitrova 2014). Consequently, it has also been a common assumption that animals have been selected to actively choose visually matching backgrounds (Kettlewell 1955; Popham 1943; Sargent & Keiper 1969; Kettlewell & Conn 1977; Caro 2005). It is somewhat surprising therefore that experimental evidence for this idea is scarce (but see Boarman et al. 1974; Gillis 1982; Sandoval 1994). Furthermore, several studies have not found support for such behaviour (Lees 1975; Merilaita, & Jormalainen 1997; Garcia & Sih 2003). Thus, even though the idea that camouflaged prey has been selected to prefer matching backgrounds has become such a common assumption, support for the idea is not as strong as often believed.

It has been proposed that visual complexity of the background is another factor that enhances prey concealment (Merilaita 2003). This idea has received experimental support from Dimitrova & Merilaita (2010, 2012, 2014), who have shown that an increase in various aspects of visual complexity of the background (complexity and diversity of shapes as well as the density of the elements that constitute the visual background) increases prey search time by blue tits (Cyanistes caeruleus). Consequently, instead of preferring visually matching background, it could be beneficial to prey to prefer visually complex backgrounds to decrease its predation risk (Merilaita 2003; Dimitrova & Merilaita 2010). However, the importance of preference for complex backgrounds has thus far not been studied. Therefore, in paper I, I investigate the importance of preference for visually matching backgrounds and preference for visually complex backgrounds by studying the background choice of the least killifish Heterandria formosa (Girard, 1859).

1.2.2 Eyespots (papers II, III and IV)
A prominent example of protective coloration is eyespots (Poulton 1890, Blest 1957a). Eyespots are patterns consisting of roughly concentric rings of contrasting colours and have received their name because to humans they often resemble the vertebrate eye. Eyespots are common in many terrestrial taxa such as insects (particularly in the order Lepidoptera), birds and reptiles, and they are also widespread in many aquatic taxa such as molluscs, flatworms and fishes (Poulton 1890; Cott 1940; Blest
Anti-predator adaptations in aquatic environments (Neudecker 1989; Meadows 1993; Stevens 2005). Because of their salience and taxonomically wide occurrence, eyespots have intrigued naturalists and biologists for more than a century (e.g. Poulton 1890). The developmental pathway and genetic basis of eyespots particularly in butterflies are now well understood (e.g. Nijhout 1991; Brakefield, et al. 1996; Beldade & Brakefield 2002; Monteiro et al. 2003). The developmental pathway of eyespots seems to be similar in fish (Ohno & Otaki 2012).

Despite the knowledge of the developmental mechanism that generates eyespots, disentangling their adaptive and functional significance has proven to be quite a challenge. It has not been until this century that rigorous, empirical support for the anti-predator functions of eyespots has started to accumulate. There are two hypotheses that are most widely-known regarding the anti-predator utility of eyespots: 1) the diversion hypothesis (also called the deflection hypothesis) and 2) the intimidation hypothesis.

1.2.2.1 The divertive effect of eyespots
The common occurrence of (generally small) eyespots found on the wing margins of many Lepidoptera and eyespots located on the caudal area in some species of fishes has been suggested to depend on the divertive function of such eyespots (Poulton 1890; Blest 1957). According to the diversion hypothesis, eyespots serve to direct the strikes of attacking predators towards less vital or defended parts of the prey body, or towards a direction that would facilitate prey escape (Poulton 1890; Blest 1957, II). For example, in a study using yellow bunting (Emberiza citrinella) as predators, and mealworms (Tenebrio molitor) with a simple spot painted onto them, Blest (1957) argued that the birds directed their attacks towards the eyespots. However, his study suffered immensely from pseudo-replication, and thus his results were not statistically sound. Despite the fact that this divertive function of eyespots was proposed over a century ago (Poulton 1890), finding empirical support for this effect has been difficult and several studies have not found any clear support for a diversion hypothesis (e.g. against birds: Lyytinen et al. 2003, 2004; lizards: Vlieger & Brakefield 2007; against fish Gagliano 2008).

During the last few years though, some support for the divertive function of eyespots has been provided in systems using passerine birds that attack butterflies or artificial prey that have marginal eyespots
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(Vallin et al. 2011; Olofsson et al. 2010; 2013). However, to date, no direct, unambiguous behavioural tests have been conducted with aquatic taxa, despite the fact that eyespots are common in aquatic environments. As previously mentioned, many species of fish have eyespots located near of in the area of their caudal fins, and these spots have been suggested to misdirect predator attacks (Cott 1940; Kelley et al. 2013; II). In paper II, I therefore investigate the behavioural response of fish towards eyespots to test for their potential divertive effect. I did this in a series of experiments in which I presented artificial prey items with or without eyespots to laboratory-reared, predator-naïve three-spined sticklebacks (Gasterosteus aculeatus). Specifically, I tested whether eyespots smaller than the attacking fish’s own eye could have a diverting effect on attacking fish, by observing where the fish directed their attacks.

Even though it has been shown that eyespots can draw the attacks of predators towards them (Olofsson 2010, 2013; Vallin et al. 2011; II), the mechanisms behind this divertive effect of eyespots remains unknown. It is still unclear whether the specific shape of eyespots is important for their function and whether eye-mimicry is involved in the divertive effect of eyespots, or could any contrasting marking have a similar effect. Paper III focuses on these questions by testing for the divertive effect of differently shaped markings that were controlled for size and contrast to the rest of the prey item. Also, this study is based on a series of laboratory experiments in which I observed where predator-naïve three-spined sticklebacks that were presented with artificial prey items directed their attacks.

1.2.2.2 The intimidating effect of eyespots

Another possible anti-predator function of eyespots that has been invoked to explain their existence in some prey is predator intimidation (Poulton 1890, Blest 1957). Generally large eyespots, for example, those found in many species of Lepidoptera, could serve to intimidate potential predators, subsequently thwart, delay or otherwise prevent an attack from being successful in favour of the prey. Recent studies have provided support for the intimidating effect of eyespots against passerine birds (Vallin et al. 2005; 2007; Stevens et al. 2007, 2008; Kodandaramaiah et al. 2009; Merilaita et al. 2011; Olofsson et al. 2013; Hossie & Sherratt 2012, 2013). However, the question why some eyespots intimidate predators has so far been unresolved and debated. Two main hypotheses for the intimidating nature of eyespots have been
proposed, one is that eyespots resemble the eyes of the predator’s own enemy (Poulton 1890; Blest 1957; Janzen et al. 2010) and hence suggest the presence of a potential threat, and the other is that the high conspicuousness of eyespots is a property that intimidates (Blest 1957; Stevens 2005; Stevens et al. 2007, 2008, 2009).

Although empirical support for the intimidating function of eyespots has been provided in terrestrial systems, surprisingly little is known about whether eyespots can have an intimidating function towards fish, despite the fact that eyespots are common also in many aquatic taxa. For this reason, in paper II, I tested whether eyespots larger than the fish’s own eye would intimidate attacking fish, by presenting three-spined sticklebacks with both spotted and spotless prey items and comparing attack latencies between these two treatment groups. Furthermore, to disentangle the importance of eye-mimicry for the intimidating function of eyespots, I studied the response of both predator-naïve, and predator-experienced three-spined sticklebacks towards prey with more or less eyelike patterns in paper IV.

1.2.3 Eyebars (paper III)
Interestingly, many species of fish, of which many also have eyespots, have a distinct stripe running vertically through their actual eye (Fig 1). Such eye stripe (or eyebar) has been suggested to serve to obscure the eyes (Thayer 1909; Cott1940; Barlow 1972; Kelley et al. 2013). Eye stripes have been suggested to disrupt the appearance of the eye by visually merging the adjacent, discontinuous surfaces of parts of the eye and the head. This particular type of disruptive coloration, which visually joins together separate and potentially revealing parts of an animal body, is called “coincident disruptive coloration” (Cott 1940; Stevens & Merilaita 2009b; Cuthill & Székely 2009). This means of concealment has received empirical support from an experiment conducted by Cuthill and Székely (2009), who pinned artificial “moths” on tree trunks and found that moths that had coincident disruptive coloration merging their body with wings survived predation by wild birds better than did moths that simply matched the background. However, there exist no experimental studies that would have demonstrated the benefit of eye-stripes. In paper III, I investigated whether an eyebar could (a) effectively disrupt an eyelike shape, (b) influence predators’ response to eyelike targets and (c) facilitate the divertive function of eyespots. I did this by observing whether an eyebar influenced where the three-spined sticklebacks
directed their attacks when presented with a prey item that had both an intact eyespot and an eyespot disrupted by a stripe.

![Copperband butterflyfish](photo.jpg)

**Figure 1.** Copperband butterflyfish (*Chelmon rostratus*) with a posteriorly located eyespot and a distinct, vertical eye stripe running through its real eye. (Photo: Karin Kjernsmo)

### 2. MATERIALS AND METHODS

#### 2.1 General methods

As my main study species I used the least killifish (*Heterandria formosa*) in paper I and the three-spined stickleback (*Gasterosteus aculeatus*) in papers II-IV. All individuals were laboratory-reared descendants of wild caught fish (one or two generations) and a more detailed description of these fish are given below. All experiments were conducted in the aquatic laboratory at the Department of Biosciences, Åbo Akademi University, Turku, Finland. I have taken utmost care to provide good attendance and environment for all the fish through the course of these studies, and all experiments were performed with permission from the Regional State Administrative Agency for Southern Finland. In each experiment of all the four studies of this thesis (papers I – IV), I have presented individual fish in 12-30-l aquaria with either
artificial backgrounds, prey items or both, and observed the behavioural responses of the fish to address my specific study questions. The artificial backgrounds and prey items used in these experiments enabled easy manipulation of their visual features, while keeping other conditions constant. Moreover, by using laboratory reared fish I could control for how much prior experience they had of predators or the stimuli they were presented with in the experiments. This would obviously have been impossible to control for, if I had used wild-caught animals.

All artificial backgrounds and prey items were created with several purpose-written programmes using the software MATLAB R2008b (The MathWorks, Inc., Natick, MA, USA), and then printed with a laser printer (HP LaserJet P4015x with 1200 dpi resolution) on water resistant paper (“Rite in the rain”, J. L. Darling Corporation, Tacoma, WA, USA). The behavioural observations of all test individuals were recorded in real time using the event-recording software J-watcher (version 1.0, available at: http://www.jwatcher.ucla.edu). Because different methodological approaches were required for each study, I below describe the methods of each study separately.

2.2 Study species

2.2.1 Heterandria formosa (paper I)

In paper I, I used the least killifish (*Heterandria formosa*) as my study species. This species can reach a body length of up to 3.5 cm, which makes it the smallest member of the Poeciliidae family. The Poeciliidae family is mainly characterized by females being livebearers, and by males having a gonopodium (modified anal fin) used for internal fertilization. The colour pattern of the least killifish consists of a light ground colour and a distinct black lateral, longitudinal stripe both in males and in females (Fig. 2). The least killifish commonly occurs in heterogeneous algal beds, where their colour patterns (i.e. the longitudinal stripe) are thought to have a concealing effect (Cox *et al.* 2009), which made them a suitable species for this study. The least killifish live in slowly moving fresh water streams or ponds, but also occur in brackish waters in southeastern parts of the USA. All least killifishes used in paper I were laboratory reared descendants from a wild population in Otter creek, Florida, USA. They were kept in 200-l (l x w h: 100 x 50 x 40 cm) aquaria, with a water temperature varying
between 24-28°C (due to weekly water changes), and held on a light/dark schedule of 16:8 hours. The least killifishes were fed 1-3 times daily *ad libitum* with live newly hatched brine shrimp (*Artemia* spp.) or commercial flake food.

![Figure 2. The study species that were used in paper I, the least killifish (*Heterandria formosa*).](image)

### 2.2.2 *Gasterosteus aculeatus* (papers II, III, IV)

For the experiments in paper II, III and IV, I used the three-spined stickleback (*Gasterosteus aculeatus*) as my study species (Fig. 3). I chose the three-spined stickleback because it is considered to be primarily a visual predator (Wootton 1976; Ohguchi 1978; Litvak & Leggett 1992; Hart & Gill 1994; Rowe *et al.* 2004), distributed over almost the entire northern hemisphere and is fairly easy to maintain in high numbers in the laboratory. To avoid any profound effects of parasitism on the behaviour of the sticklebacks, I derived laboratory populations from parental fish caught outside Tvärminne zoological field station in South-western Finland (59° 50’ N, 23° 12’ E) through artificial fertilization. It has been shown that for example the common tapeworm, *Schistocephalus solidus*, can affect their behaviour drastically (eg. Giles 1987, Godin & Sproul 1988, Barber & Huntingford 1995). Also, another benefit of using laboratory reared fish is that they are certain to be naïve to both predators and to prey with eyespots, which is why I have been able to study their innate behavioural response towards the prey items.

![Figure 3. The three-spined stickleback (*Gasterosteus aculeatus*) that was used as study species in papers II-IV.](image)
2.3 Background choice as an anti-predator strategy (paper I)

As mentioned in the introduction, it has traditionally been assumed that to decrease its predation risk, a prey should prefer habitats that are visually similar to its colour pattern. This hypothesis has been challenged by a hypothesis according to which a preference for visually complex backgrounds provides another way to decrease predation risk and that this could be even more beneficial than preference for matching backgrounds. Visual background complexity has been shown to decrease the risk of being detected by predators (Dimitrova & Merilaita 2010, 2012). Moreover, it may be cognitively more demanding to identify visually similarity (that requires a comparison) than visual complexity. To study the relative importance of background matching and visual complexity for the background choice of the least killifish, I therefore conducted a series of controlled laboratory experiments and presented the fish with artificial backgrounds that had varying patterns. I also manipulated the levels of predation threat (predator present or absent) to establish whether the observed behaviour is an anti-predator response. To simulate predation risk, I used convict cichlids (*Amatitlania siquia*) as predators. Convict cichlids are substantially larger than the least killifish, and can reach a total length of 12 cm.

The pattern of the least killifish (Fig. 1) enabled me to create artificial backgrounds with black elements that approximately matched the shape and the area of the lateral stripe of the fish. The stripes in the backgrounds had the same average size as the least killifish’s stripes. A total of four differently patterned backgrounds were produced: one with matching pattern, one with a mismatching pattern, and two different types of complex patterns (Fig. 4), and all of them had identical black to white ratio, consisting of 22% black.

Two of these four backgrounds at time were used over the course of three different experiments. First, the fish was given a choice between the matching (horizontal) and mismatching (vertical) stripes (Fig. 3a and b, respectively). In the second experiment, the matching stripes was presented together with a background consisting of the size- and shape-matching stripes, but now with added complexity through random orientation and by allowing overlap between the randomly distributed stripes (Fig. 3c). This overlap decreased the match between the prey and this background pattern, but it also increased the visual complexity (i.e.,
the perimeter-to-area ratio, Dimitrova & Merilaita 2012) of the shape of the background pattern. In the third experiment of this study, the matching background was presented together with a background consisting of non-overlapping elements with increased complexity of shape compared to the stripes (Fig. 3d). In each aquarium, the two backgrounds covered exactly 50% each of the sides.

Figure 4. Sample of the four different background patterns used in the background choice experiment. a) The background pattern with matching, horizontally oriented stripes, b) otherwise similar stripes but with a mismatching, vertical orientation, c) otherwise similar stripes but with complex orientation and overlap allowed and d) the background consisting of the complex shapes.

In the beginning of each replicate, I placed a randomly chosen killifish in the middle of the experimental 12-l aquaria. I ran half of the replicates with simulated predation threat. For the predation treatment, I put a convict cichlid in a cylindrical container made of transparent plastic and mesh (diameter 10 cm, height 15 cm), and placed it in the middle of the aquarium. After the focal fish had had two minutes to calm down, I observed each replicate for 15 minutes, recording the background choice of the fish. An equal number of females and males were used in each treatment group, and each individual were only tested once.

2.4 Divertive eyespots (papers II, III)
With these studies, I investigated the anti-predator function of eyespots and tested for the importance of the divertive effect in aquatic environments. More specifically, I studied if eyespots could be used to manipulate where attacking fish direct their strikes, and whether this divertive effect is affected by the visual properties, such as the eyelike shape of the marking. In these experiments I presented predator-naïve, laboratory reared three-spined sticklebacks (Gasterosteus aculeatus) with artificial prey items and recorded their response towards prey that had different markings.
I first trained individual three-spined sticklebacks to attack the artificial prey items and then studied their response to the patterning of the experimental prey items (Fig. 5). All prey items consisted of two components, a rectangular piece of printed paper that covered a thawed red mosquito larva (*Chironomidae* sp.) which served as a reward and ensured that the fish would be motivated to attack the artificial prey items. Depending on the experiment, each rectangular prey item had either: one eyelike or non-eyelike mark on only one half of its upper surface, two different marks (i.e. one on either half), or no mark if it was a control prey (Fig. 5). Each prey item was produced by printing different black patterns onto white paper, and consisted of different conspicuous markings that were controlled for size and contrast to the rest of the prey item. In all experiments, I used eyespots as well as non-eyelike marks and tested if they diverted the attacks of the sticklebacks by observing where the fish directed their attacks. In the last experiment of paper III, I studied if an eyebar could be used to effectively disrupt an eyelike shape and thus also influence predators’ response to the shape. In the latter experiment, I presented the fish with prey items that had both an intact eyespot and an eyespot disrupted by an eyebar (Fig. 4).

![Figure 5](image)

**Figure 5.** The prey items used to study the divertive effect of prey markings. a) A single eyespot, b) an eyespot together with a square-shaped element, c) a single square, d) an intact eyelike mark and an eyelike mark disrupted by a bar, e) the control prey with only the mottled background pattern.

Training and experiments were conducted in 30-l aquaria that were lit from above by natural light simulating fluorescent lamps. Each aquarium consisted of three main zones, the start zone that was separated from the mid zone by a removable opaque plastic divider, and
the foraging zone where the foraging plate (9.5 x 7 cm in size) and prey items was placed (Fig. 6).

![Figure 6. Schematic figure of the experimental aquarium. The fish was initially put in the start zone (SZ), separated by an opaque divider from the mid zone (MZ) and the foraging zone (FZ). FP is the foraging plate where the artificial prey item was placed.]

There was only one fish in each experimental aquarium. However, each individual was tested four times to establish how repeated encounters with the prey influenced the behaviour of the fish. At the beginning of an experimental trial, the fish was in the start zone, and a prey item was placed in the foraging zone two minutes before the trial started. When a trial started, the divider was lifted to give the fish access to the foraging zone. A trial lasted until a fish attacked the prey item or the maximum observation time of 15 minutes had passed.

2.5 Intimidating effect of eyespots and the importance of eye mimicry

Because it has been shown that eyespots have an intimidating function in terrestrial systems, and large eyespots are common in the aquatic environment as well, I tested whether eyespots could have an intimidating function against attacking fish. More specifically, I investigated whether an eyespot larger than the fish's own eye would intimidate potential predators, by presenting the fish with either a prey item that had a large eyespot, or a spotless prey item, and compare attack latencies between these two (paper II). The experimental
procedure followed that of paper II and III, i.e. the fish was first trained to search for artificial prey items, and then I observed the response of the fish towards the experimental prey items.

Although there is convincing support for the intimidating effect of eyespots, it has been unclear what causes this effect. So far no direct evidence has been provided for the idea that predators do associate eyelike shapes with a threat from their enemies, despite the fact that this has been the most popular hypothesis invoked to explain this effect. To investigate the importance of eye mimicry for the intimating effect of eyespots, I therefore tested the behavioural response of both predator-naive and predator-experienced three-spined sticklebacks (*Gasterosteus aculeatus*) to artificial prey items that had marks that were either eyelike, being either similar to or different from the typical eyespot shape, or non-eyelike (paper IV). One key assumption for the approach I chose for this study is that if eye mimicry is important for the intimidating effect of eyespots, then not only displays based on the orthogonal projections of the eye, resembling the typical shape of eyespots, but also displays based on other projections of the eye should intimidate predators.

Following the procedure of paper II and III, I derived sticklebacks fry from wild-caught parental fish through artificial fertilization (Kjerns mo & Merilaita 2013). To manipulate predator-experience, I then randomly divided the stickleback fry into two long term pre-experiment treatment groups. One group was subjected to visual and olfactory cues of perch (*Perca fluviatilis*), which is a natural predator of three-spined sticklebacks. The other group reared in the absence of any cues of predators.

After the sticklebacks had been exposed to the pre-experiment treatment for at least half a year, I tested their behavioural response to the different prey items. Each prey item consisted of two components, a rectangular piece of paper (Fig. 7), and a frozen red chironomid larva that was placed on the middle of the prey item. Four different types of prey items were created containing either: 1) one mark mimicking the lateral view of a perch eye, 2) two marks mimicking the frontal view of a perch eyes, 3) a non-eyelike rectangular mark that had equally large black and white areas as the single eyelike mark to control for the level of contrast and conspicuousness or 4) only the mottled background patterning (Fig. 7). The eye-mimicking patterns were reproduced from photographs of perches’ eyes.
In the experiment, a randomly chosen fish from the pre-experiment treatment were placed in the start zone of a 30-l experimental aquarium, behind a removable opaque plastic divider that separated the start zone from the rest of the aquarium. One out of four different prey items were then placed on a foraging plate in the foraging zone of the aquarium (Fig. 6), and the observation of each fish then started by lifting the divider. I recorded the latency to prey attack (defined as the time it took for a fish to attack the prey item from initiating prey approach i.e. when positioned above the foraging plate face down towards the prey) by using the same event-recording software as in papers I, II and III.

3. RESULTS AND DISCUSSION

3.1 Background choice as an anti-predator strategy (paper I)

3.1.1 Predator induced preference for background-matching pattern
When testing for choice between matching and mismatching background pattern, neither male nor female least killifish showed any background preference in the absence of a predator (Fig. 8a). Interestingly, however, under simulated predation threat both males and females a significant preference for the matching background before the mismatching background (Fig. 8b). These results provide empirical support for the idea that prey could be selected to prefer habitats that yield protection through improved background matching (Kettlewell 1955). Furthermore, the results also suggests that the least killifish are able to assess and respond to some cues related to visual similarity.
between their body pattern and the background, and that those cues serve as indicators for habitat safety.

**Figure 8.** Proportion of time (mean ± s.e.) spent on the matching background (bkgd) in both males and females. Panels a, c and e show background choice without predation threat and panels b, d and f show background choice under simulated predation threat. N = 15 in each group for the matching vs. mismatching treatment, and N = 20 each for all the other groups. The dashed line indicates the expected no choice value of 50%. For each treatment, I tested whether time spent in the matching background deviated from the 50% expectation using the t-test (***, p < 0.001; **, p < 0.01; *, p < 0.05; n.s.: non-significant).
3.1.2 Preference for visual complexity differs between males and females

In the second experiment, in which the fish were presented with a choice between the matching and the more complex background with matching but randomly-rotated, overlapping stripes, neither females nor males showed any preference for either background in the absence of predator (Fig. 8c). In the presence of a predator, females did, however, prefer the complex background before the matching background, whereas males showed no preference (Fig. 8d). For males, this could be either because they found both backgrounds equally protective, or because they found them equally unprotective. The first alternative seems more likely because males displayed a strong preference for the matching background when given a choice between the matching- and the mismatching pattern.

In the third experiment where the fish were presented with a choice between the matching background and the background consisting of complex-mismatching pattern elements, males and females again behaved differently. Males spent significantly more time in the matching background than in the complex background, regardless of predator presence (Fig. 8e, f). Females, on the other hand, did not show a preference for either background (Fig. 8e, f). This may indicate that the males experience that the complex background deviated too much from the males’ own pattern or that the level or type of complexity was not protective enough, whereas the females experienced them equally protective.

Both a visual match between prey colour pattern and the background and visual complexity of the background are known to decrease predation risk (Kettlewell 1955; Feltmate & Williams 1989; Merilaita 2001; Dimitrova & Merilaita 2010, 2012). Collectively, my results show that in the least killifish, background matching is an important aspect in background choice, but also some aspects of complexity are important and may even override background matching. Particularly in visually patchy or variable habitats, visual complexity may often be a less specific requirement and therefore more likely to come across than a visual match between a specific pattern and background. Visual complexity could therefore serve in many habitats as a simpler and more straightforward cue of a protective background.

Background choice differed to some extent between sexes, such that in general females preferred the more complex backgrounds more than
males did. This suggests that male and female least killifishes have different background choice or habitat use strategies, as previously suggested in many other species (Shine 1986; Asakura 1995; Merilaita & Jormalainen 1997, 2000). Since female least killifishes are larger than males, they probably also have higher resource needs (Alcock 2005), which suggest that opportunity costs for background matching (Ruxton et al. 2004) could be higher for females than males. To decrease this cost, females could therefore benefit from preferring visual complexity over background matching if it expands the range of microhabitats where they can forage safely. Similarly, the smaller size in males suggests that males could acquire their necessary resources by utilizing a smaller range of microhabitats than females, which results in lower opportunity costs for background matching in males and could explain why males generally showed preference for the matching background.

3.2 Divertive eyespots (papers II, III)

3.2.1 Eyespots divert attacks by naïve fish, whereas less eyelike marking shapes do not

When presented with a prey item that had one small eyespot on one half of the prey, significantly more of the sticklebacks directed their first attacks towards the eyespot compared to the spotless side of the prey ($\chi^2 = 11.64$, d.f. = 1, $p < 0.001$). This is an important finding, because it provides empirical support for the idea that eyespots can draw the attacks of predators towards them and thus, manipulate predator behaviour. Compared to previous studies, this effect seems strikingly strong, and to my knowledge, this is also the first time that unambiguous, empirical support for divertive effect of eyespots has been shown in aquatic taxa.

Interestingly, when the eyespot was replaced with an equally conspicuous, square-shaped mark, there was no divertive effect on the first attack of the fish ($\chi^2 = 0.20$, d.f. = 1, $p = 0.65$). Also, when the prey had both an eyespot and the square-shaped marking, the markings did not influence to which half of the prey the fish directed their first attack. However, when the prey had both an intact eyespot, and an eyespot disrupted by a bar, significantly more of the sticklebacks directed their first attack towards the intact eyespot ($\chi^2 = 11.27$, d.f. = 1, $p < 0.001$).

These results are in accordance with some other recent studies that have shown that eyespots can be used to divert attacks by predators.
Particularly the result of the last experiment suggests that eyelike shapes are especially effective divertive marks. The finding that divertive effect of an eyelike shape could be manipulated with an eyebar supports the principle of coincident disruptive colouration (Cott 1940; Cuthill & Székely 2009). In addition, this result suggests that the relatively common occurrence of eyebars in fishes could be explained by increased camouflage or, in species that have eyespots, amplified divertive effect through concealment of the eye.

3.3 Intimidating eyespots and the importance of eye-mimicry (papers II, IV)

The experiment in paper II did not provide any clear support for intimidating effect of eyespots towards attacking fish. When comparing attack latencies between the spotless prey and the prey that had a large eyespot, there was no significant difference in any of the four trials (Cox regression, trial 1: Wald = 0, n = 41, p = 0.98; trial 2: Wald = 2.91, n = 41, p = 0.09; trial 3: Wald = 1.98, n = 41, p = 0.16; trial 4: Wald = 1.03, n = 41, p = 0.31). This result was somewhat surprising, considering the strong support the intimidating function of eyespots has received in terrestrial studies using passerine predators and butterflies as prey (Vallin et al. 2005, 2006, 2007, Kodandaramaiah et al. 2009, Merilaita et al. 2011), and that eyespots have been suggested to have an intimidating effect in fish as well (Coss 1979; Neudecker 1989).

As opposed to the lack of support in paper II, the setup of paper IV provided clear support for intimidating effect of eyelike patterns towards attacking fish by demonstrating that eyelike prey patterns induced hesitant behaviour in the sticklebacks whereas non-eyelike patterns did not have such effect (Fig.9). Both the predator-naïve sticklebacks (two-sample t-test: t = 4.20, d.f. = 28, p < 0.001) and the sticklebacks that had been subjected to visual and olfactory cues of predators (t = 5.25, d.f. = 28, p < 0.001) were more hesitant to attack the prey that had an eyelike mark than the prey that had a non-eyelike mark. Moreover, sticklebacks that had experience on predator cues took significantly longer to attack the prey item that had the single eyelike mark that resembled a typical eyespot, than the prey item that had the equally conspicuous, but non-eyelike rectangular marking (t = 5.25, d.f. = 28, p < 0.001). Importantly, predator-experienced fish also took longer to
attack the prey that had two eyelike marks (that imitated the frontal view of perch eyes and that did not resemble the typical eyespot shape) compared the prey that had a rectangular, non-eyelike marking ($t = 4.72$, d.f. = 28, $p < 0.001$).

![Figure 9](image)

**Figure 9.** Mean attack latencies (± s.e.) for fish that attacked the eyelike patterns are shown in a) and b), and c) show the results from the non-eyelike, rectangular element. P is the pre-experimental “predation” treatment and N represents “no predation”. N = 15 for each group.

These results support the suggestion that eye mimicry is important for the intimidating effect of eyespots for two reasons. 1) The sticklebacks took significantly longer time to attack the prey that had eyelike markings than the prey that had the equally conspicuous, rectangular marking. 2) Previous experience of predators only affected the response of the sticklebacks to the eyelike markings but not to the non-eyelike markings, suggesting that predator cues induced an association between the markings and a threat. These results contradict previous studies arguing that it is merely the conspicuousness, and not eye mimicry that is important for the intimidating function of eyespots (Stevens *et al*. 2007, 2008, 2009).

Considering that both the eyelike markings resembling the typical eyespot shape and the eyelike markings that deviated from the concentric, circular shape induced an intimidating effect towards attacking fish, it is interesting that most eyespots found in many fishes are more or less circular (e.g. Kelley *et al*. 2013). The appearance of eyespots could be explained by the developmental mechanisms that can relatively easily produce circular patterns that consist of concentric rings (Beldade & Brakefield 2002; Dilão & Sainhas 2004).
4. GENERAL CONCLUSIONS

The adaptive value and function of both cryptic colouration and eyespots have received much attention and have been studied particularly in terrestrial systems (e.g. Stevens & Merilaita 2011; Vallin et al. 2005, 2006; Kodandaramaiah et al. 2009), but far less is known about them in aquatic systems, despite the fact that probably all types of protective coloration found in terrestrial environments also exist in the aquatic environment and have initially evolved there. In this thesis, I have used aquatic experimental setups to study specific questions about the functions and adaptive value of protective coloration and related anti-predator behaviour in aquatic environments, and showed that the visual appearance of backgrounds and prey colour patterns indeed strongly affects the behaviour of fishes.

More specifically, in paper I, I have shown that certain aspects of the visual appearance of the available backgrounds are important for the habitat choice of fish and that this is related to predator avoidance. Because it has been shown that both a visual match in the appearance of prey colour pattern and background, and visual complexity of the background can decrease the risk of predation (Feltmate & Williams 1989; Kettlewell 1955; Dimitrova & Merilaita 2010, 2012), my finding that fish showed a preference for these cues when under predation risk suggests that this behaviour is indeed an anti-predator adaptation. Intriguingly, the latter finding also suggest that prey are able to assess and respond to some cues of visual similarity between their body pattern and the surrounding environment, but also that some aspects of visual complexity could be important and may even override background matching. Because visual complexity might be an easier and more straightforward cue than background matching to find in heterogeneous environments, future studies should investigate whether increased visual variability of the habitat leads to an increased preference for visual complexity, and try to pinpoint which aspects of visual complexity are important for habitat choice of prey.

In papers II, III and IV, I have shown that eyelike prey markings can be used to manipulate predator behaviour in a way that reduces the likelihood of a successful attack, hence providing adaptive explanations for the wide occurrence of eyespots in many prey species. The results from papers II and III show that eyespots very effectively draw the attacks of fish towards them, providing strong support for Cott’s (1940)
suggestion that the existence of eyespots in many fishes can be explained by their divertive function. Moreover, the finding that circular, possibly eyelike marks drew the initial attacks of fish towards them more effectively than did marks of other shapes may at least partly, probably together with the relatively simple developmental process underlying circular marks, explain why divertive eyespots have their circular appearance. It is possible that eyelike shape is an important factor for the divertive effect due to eye mimicry in aquatic prey (Winemiller 1990). Although it is often assumed that divertive eyespots, particularly in Lepidoptera, draw attacks away from vital parts of the body, in many aquatic prey eyespots could instead serve as a false indicator of expected escape direction. This could explain why many species of fish have eyespots located near, or on their caudal fins. A posteriorly-located eye-mimicking mark may deceive a predator to attack towards that direction, and that way enhance the probability of a successful escape.

Paper III confirms the anti-predator utility of another common prey pattern, eyebar. My results show that a bar effectively disrupts an eyelike shape. Also the disruption of the eyelike shape resulted in a significant amount of attacks being directed towards the intact eyelike shape. This result therefore suggests functionally important things. First, eyebars provide an effective way to conceal the eye, which may be an important means of camouflage as the eye can serve as a cue for detection and recognition of potential prey. Moreover, in prey that has a divertive eyespot, the eyebar can strengthen the divertive effect of the eyespot, which might explain the joint occurrence of eyebars and eyespots in many species of fish.

I found no clear support for an intimidating effect of eyespots in paper II. It might be that the methodological approach I used to test for the intimidating effect of eyespots was not properly designed for testing this. Because I only measured the latency to attack from the fish’s last entry in the foraging zone, and that some individuals swam around more than others before attacking the prey item, it might be that I failed to measure some important behavioural response that occurred after the fish’s entry in the foraging zone, but before the prey attack.

On the contrary however, I found strong support for an intimidating effect of eyelike prey markings towards attacking fish in paper IV. Importantly, I have provided empirical evidence for the idea that eye mimicry, and not merely conspicuousness, is an important reason of this
intimidating effect. This experiment also revealed that the intimidating effect is partly innate and partly acquired through previous experience of predators.

To conclude, I have in this thesis presented adaptive explanations for the existence of eyespots, eyebars and background choice in prey, and that way increased our understanding of how natural selection imposed by predation can shape the appearance and anti-predator behaviour of prey over evolutionary time. Also, my finding that manipulating the visual aspects of prey appearance strongly influenced the behavioural response of predators has provided important knowledge in questions regarding predator psychology.
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Karin Kjernsmo

Anti-predator Adaptations in Aquatic Environments

This thesis investigates how protective coloration can be used, either as such or together with behavioural adaptations, to manipulate predator behaviour and decrease predation risk. The results demonstrate that protective coloration provides numerous distinctive ways for aquatic prey to escape predation. Thus, visual perception and behaviour of fish are important factors shaping the appearance and behaviours of aquatic prey.