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RESEARCH ARTICLE

Experimental manipulation of perceived predation risk and cortisol generates contrasting trait trajectories in plastic crucian carp

Jerker Vinterstare[1,*], Kaj Hulthén[1], P. Anders Nilsson[1,2], Helen Nilsson Sköld[3] and Christer Brönmark[1]

ABSTRACT

Most animals constitute potential prey and must respond appropriately to predator-mediated stress in order to survive. Numerous prey also adaptively tailor their response to the prevailing level of risk and stress imposed by their natural enemies, i.e. they adopt an inducible defence strategy. Predator exposure may activate the stress axis, and drive the expression of anti-predator traits that facilitate survival in a high-risk environment (the predation–stress hypothesis). Here, we quantified two key morphological anti-predator traits, body morphology and coloration, in crucian carp reared in the presence or absence of a predator (pike) in addition to experimental manipulation of physiological stress via implants containing either cortisol or a cortisol inhibitor. We found that predator-exposed fish expressed a deeper-bodied phenotype and darker body coloration as compared with non-exposed individuals. Skin analyses revealed that an increase in the amount of melanophores caused the dramatic colour change in predator-exposed fish. Increased melanization is costly, and the darker body coloration may act as an inducible defence against predation, via a conspicuous signal of the morphological defence or by crypsis towards dark environments and a nocturnal lifestyle. By contrast, the phenotype of individuals carrying cortisol implants did not mirror the phenotype of predator-exposed fish but instead exhibited opposite trajectories of trait change: a shallow-bodied morphology with a lighter body coloration as compared with sham-treated fish. The cortisol inhibitor did not influence the phenotype of fish i.e. neither body depth nor body coloration differed between this group and predator-exposed fish with a sham implant. However, our results illuminate a potential link between stress physiology and morphological defence expression.

KEY WORDS: Inducible defences, Phenotypic plasticity, Stress, Stress axis, Colour change

INTRODUCTION

Almost all animals constitute potential prey and, hence, predation is a central feature of most natural systems. As failure to avoid a potential predator is definitive (i.e. leads to death), prey demonstrate a plethora of anti-predator defences to avoid capture and consumption (Brodie, 1977; Brönmark and Miner, 1992; Cott, 1940; Hodge et al., 2018; Price et al., 2015; Ydenberg and Dill, 1986; Young et al., 2004). Over time, anti-predator traits and defence phenotypes may become canalized (Waddington, 1959), and thus are always expressed regardless of the prevailing predation risk (e.g. Välimäki et al., 2012). However, spatio-temporal variation in predation risk is common, and under such circumstances defence phenotypes can be the result of within-generation developmental responses to environmental cues, i.e. predator-induced plasticity or inducible defences (Tollrian and Harvell, 1999). By adopting an inducible strategy, prey can achieve a closer phenotype–environment match, and the energy allocated to build and maintain a defence can be saved when predators are absent (Dewitt et al., 1998; Kishida and Nishimura, 2006; McCollum and Van Buskirk, 1996; Tollrian and Harvell, 1999). Multiple inducible defence strategies have been identified, including modification to behaviours (Fraker, 2008; Höglund et al., 2005; Hulthén et al., 2015; Pettersson et al., 2000; Relyea, 2003), morphology (Brönmark and Miner, 1992; Van Buskirk et al., 1997), physiology (Clinchy et al., 2013; Furtbauer et al., 2015; Maher et al., 2013; Sapolsky et al., 2000), life-history (Kusch and Chivers, 2004; Pollock et al., 2005) and body coloration (Ahlgren et al., 2013; Cortesi et al., 2015). While many studies have focused on how predator exposure can alter the expression of a single trait, few studies have investigated whether prey facultatively alter multiple and disparate trait types. Furthermore, there is a critical gap in our knowledge regarding the proximate mechanisms underlying the regulation and expression of plastic anti-predator defence phenotypes.

A ubiquitous anti-predator response among vertebrates is the neuroendocrine hypothalamus–pituitary–adrenal/interrenal (HPA/HPI) axis, commonly referred to as the stress axis (Clinchy et al., 2013; Hammerschlag et al., 2017; Oliveira et al., 2014; Sapolsky et al., 2000). Numerous studies have examined its function in prey upon predator exposure, where the physiological response shows as increased secretion of glucocorticoids, e.g. corticosterone or cortisol (Clinchy et al., 2013; Hammerschlag et al., 2017; Oliveira et al., 2014; Sapolsky et al., 2000). Recently, it was demonstrated that an increase in glucocorticoid concentration, caused by either predator presence or experimental corticosterone manipulation, triggers facultative expression of a larger tail in amphibian tadpoles (Maher et al., 2013). Larger tails enhance fast-start performance, resulting in increased survival probability when attacked by predators, a well-studied example of an adaptive inducible defence trait in tadpoles (McCollum and Van Buskirk, 1996; Relyea, 2003; Van Buskirk et al., 1997). Interestingly, the authors also reported that the distribution of metyrapone (a glucocorticoid inhibitor) into experimental tanks containing a natural predator reduced the magnitude of defence expression, further highlighting the potential role of stress hormones in regulating the expression of inducible defence traits (Maher et al., 2013).

Here, we quantified facultative trait expression in crucian carp (*Carassius carassius*) in response to experimental manipulation of

[1]Department of Biology, Aquatic Ecology Unit, Ecology Building, Lund University, 223 62 Lund, Sweden. [2]Department of Environmental and Life Sciences, Karlstad University, 651 88 Karlstad, Sweden. [3]Havets Hus in Lysekil, Strandvägen 9, 453 30 Lysekil, Sweden.

*Author for correspondence (jerker.vinterstare@biol.lu.se)

J.V., 0000-0001-6998-4632; K.H., 0000-0003-4360-5555; P.A.N., 0000-0002-3541-9835; C.B., 000-0002-5203-8727

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perceived predation risk (presence or absence) and cortisol (cortisol or metyrapone). We quantified changes in crucian carp relative body depth, a key predator-induced trait known to reduce predation risk in our model organism (Brönmark and Miner, 1992; Nilsson et al., 1995). The change from a shallow-bodied to a deep-bodied phenotype reduces predation risk as deep-bodied individuals are more difficult to capture because of their enhanced escape performance and aggravated handling for gape-limited predators (Domenici et al., 2008; Nilsson et al., 1995). Furthermore, we analysed changes in body coloration as this trait is known to play an integral role in communication and predation avoidance (Caro et al., 2016; Cortesi et al., 2015; Schweitzer et al., 2015; Stuart-Fox and Moussalli, 2009). Despite this, few studies have quantified whether individuals can facultatively adjust their colour in response to perceived predation risk and stress. Teleost fish can change external body coloration via two different mechanisms, 'physiological' colour change acting through a synchronized transport of the pigment inside chromatophores (Fujii, 2000; Sköld et al., 2013), and 'morphological' colour change regulated via chromatophore density and shape, and the amount of internal pigments (Leclercq et al., 2010; Sugimoto, 2002). The former is rapid, occurring within minutes, whereas the latter takes substantially more time, usually weeks or months, to occur (Bagnara and Matsumoto, 2006; Sugimoto, 2002). Further, a physiological colour change may eventually develop into a morphological colour change if the triggering stimuli are chronic and act over longer time scales (Bagnara and Matsumoto, 2006; Sugimoto, 2002). Given that many different defence traits may combine to produce an integrated anti-predator defence phenotype (e.g. Pigliucci and Preston, 2004), we predicted both morphology and colour would respond to manipulations to the predator environment. For example, a darker body coloration may enhance the silhouette of a deep-bodied and hard-to-capture prey phenotype by producing greater contrast, i.e. be an adaptive signal to potential predators (Caro, 2009; Rojas et al., 2015). A darker body coloration may also be of adaptive value for crypsis and act in concert with the shift towards more nocturnal activity in crucian carp under intense predation risk (J.V., K.H., D. E. Nilsson, P.A.N. and C.B., unpublished data). Furthermore, if activation of the physiological stress response underlies the expression of inducible anti-predator traits in crucian carp, we also predicted that experimental manipulation using cortisol implants would produce a deeper-bodied and darker phenotype similar to the one expressed following exposure to real predators, as recently shown in amphibian tadpoles (Maher et al., 2013). To test these hypotheses, we performed controlled experiments where we manipulated predation risk (predator presence/absence) and the stress axis (via intraperitoneal implants containing either cortisol or metyrapone). After 135 days of treatment exposure, we used digital photography and chromatophore analyses to quantify treatment effects on body morphology and coloration traits.

MATERIALS AND METHODS

Study organism

Crucian carp, *Carassius carassius* (Linnaeus 1758), a common freshwater fish, constitute an ideal vertebrate model to study inducible defence expression in response to predation risk. Multiple field and laboratory experiments have demonstrated that crucian carp respond to chemical cues from predatory fish, such as pike (*Esox lucius*), by inducing a deeper body shape (Brönmark and Miner, 1992; Brönmark and Pettersson, 1994) and by shifts in key behaviours, such as becoming more bold and less active when the perceived risk of predation is high (Höglund et al., 2005; Hulthén et al., 2014; Pettersson et al., 2000).

Fish collection and experimental set-up

Wild, predator-naive crucian carp were caught with fyke nets between 16 and 23 July 2014 in a 0.06 ha pond containing only crucian carp, located ∼18 km from the experimental facility at Lund University, southern Sweden (pond: 55°42′34.4″N 13°27′18.5″E). Experimental fish [n=144, body mass (M_b): 9.3±0.9 g; mean±s.d.] were haphazardly distributed into 24 identical experimental tanks (152 l, 95×40×40 cm, n=6 crucian carp per tank). Tanks contained aerated tap water that was filtered through a 10 cm thick foam sponge filter in one end of each aquarium, and all tanks were divided in half by a transparent and perforated acrylic glass divider, thus allowing crucian carp in the predator treatments to perceive both visual and chemical cues from the pike held in the other compartment of the tank, but eliminating the risk of actual predation. To prevent visual interactions between replicate tanks, a black non-transparent plastic film was externally attached to the open side of each tank (opposite to the filter side). Prior to the experimental period, all fish were removed from the tanks, anaesthetized with benzocaine and surgically implanted with a small passive integrated transponder (PIT) tag (HDX, Oregon RFID, 12.0 mm long and 2.12 mm diameter, mass 0.1 g) into the abdominal cavity (Skov et al., 2005), allowing individual fish identification over the whole experimental period. Light and temperature were held constant during the experimental period, fixed to 14 h light:10 h dark (light from 06:00 h until 20:00 h) and 18°C. Experimental pike were caught by electrofishing in lake Krankesjön, situated ca. 500 m from the crucian carp pond. Experiments were performed under permission from the Malmö/Lund authority for ethics of animal experimentation (licence M182-15).

Experimental treatments

Experimental tanks were assigned (random permutation) to one of four treatments: predator absent with sham implant (Sham), predator absent with cortisol implant (CORT), predator present with sham implant (P+Sham) and predator present with metyrapone implant (P+MTP). Metyrapone, here used as a cortisol blocker, acts by inhibiting the synthesis of cortisol from 11-deoxycortisol by 11β-hydroxylase, a biomedical process that has been demonstrated to successfully inhibit cortisol synthesis in teleost fish (Hopkins et al., 1995; Milligan, 2003; Rodela et al., 2012). On 10 December, all fish were weighed (to the nearest 0.1 g) to enable calculation of the total volume of substances needed to produce implants suitable for body mass-specific delivery (see 'Implants', below). The experimental period was initiated 12 days later (on 22 December) when fish received the treatment-specific implant as described above, and a single pike (size range 24.5–27.9 cm) was added to each tank for the two predator treatments. Implants were injected intraperitoneally with a 23-gauge needle inserted posterior to the pectoral fin. After each injection, we immediately placed a small ice bag on the injection site to enhance solidification of the implants, following previous work (e.g. Bernier and Peter, 2001). Fish that died within the first 8 days of the experiment (n=6) were replaced with implanted fish of similar size, while we only added fish to maintain density levels if mortality occurred later. Mortality rates over the course of the experiment varied between treatments (number of cases of mortality for the different treatments: Sham N=0, CORT N=8, P+Sham N=4 and P+MTP N=5; Fisher–Freeman–Halton test: $\chi^2_{3,n=144}$=0.81, P=0.016), but this difference

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was driven by the complete absence of mortality in the Sham treatment, whereas no difference in mortality was found between the other three groups $\chi^2_{2,n=108}=1.815$, $P=0.404$). Experimental subjects were fed a mix of frozen chironomids and carp pellets, at a delivery rate corresponding to 3% of the total M_b within each experimental tank.

Implants

Cortisol (hydrocortisone, USP 1316004) or metyrapone (Sigma-Aldrich M2696) was mixed with melted cocoa butter (ClearLife) as a vehicle for prolonged substance distribution, following previous studies that manipulated the stress axis in teleost fish (e.g. Bernier et al., 2004; Bernier and Peter, 2001; Carragher et al., 1989; Lawrence et al., 2017; McConnachie et al., 2012; Midwood et al., 2014; Pickering et al., 1989). When the substances were completely dissolved in the melted cocoa butter (∼40°C), implant solutions were immediately transferred to syringes (1 ml, TERUMO) and stored at 4°C until the time of injection. As we focused on the effects of cortisol implants on the expression of anti-predator traits, and had no direct interest in treatment-specific plasma concentrations of cortisol, we employed dosages of cortisol and metyrapone that previously have been shown to affect plasma levels of cortisol in *Carassius* species: 150 µg cortisol g^{-1} M_b (e.g. Bernier et al., 2004) and 200 µg metyrapone g^{-1} M_b (e.g. Bernier and Peter, 2001), each mixed in 10 µl cocoa butter. All substance deliveries, including the sham injections (containing plain cocoa butter), were individually adjusted to correspond to an intraperitoneal injection of 10 µl implant solution g^{-1} M_b. Fish body mass did not differ among treatments at the start of the experiment (ANOVA; $F_{3,21.02}=0.24$, $P=0.869$).

Morphology and colour quantification

We terminated the experiment 135 days post-implantation, when all fish were laterally photographed to provide digital images for subsequent analyses of relative body depth and body coloration (percentage of black pixels). Moreover, one fish per tank was killed for another study, 13 days prior to the end of the experiment; these fish were also pooled in the total sample size and included in all statistical analyses. A digital single lens reflex (DSLR) camera (EOS 450D, Canon Inc., Tokyo, Japan) equipped with an 18–35 mm lens (f/3.5–5.6 IS) was vertically mounted on a copy-stand. All images were captured in a standardized light environment using a closed light tent photo studio and four external light bulbs horizontally projected towards the tent. Each image was captured remotely using a Canon RC-6 controller. The fish were netted from their home tanks and laterally placed on a glass plate located on a white foam board centred below the lens of the camera. A ruler and a black and white colour card standard were included in each image for subsequent calibration of scale and white balance.

Morphological measurements

From the digital images, we extracted morphological variables using the image analysis software ImageJ v.1.49 (https://imagej.nih.gov/ij/). Standard length was measured as the distance between the tip of the snout and the end of the last scale anterior to the caudal fin. To exclude potential effects of differences in body condition and only account for the expression of the morphological defence, body depth was measured as the vertical distance from the anterior insertion part of the dorsal fin to the lateral line (Vinterstare et al., 2019). Moreover, we calculated Fulton's condition factor K ($K=M/L_S^3$, where M is the mass and L_S is the standard length for each fish at the end of the experiment) in order to test for differences in general body condition between treatments. No differences in body condition were found (nested one-way ANOVA: $F_{3,20.59}=2.09$, $P=0.133$).

Colour analysis

Digital photography is an established method to quantify and compare animal coloration, and here, we followed an earlier established method (Ahlgren et al., 2013; Rodgers et al., 2013, 2010; Touchon and Warkentin, 2008). In brief, all images were first converted to 8-bit binary pictures so that each image ranged from 0 to 255 shades of grey, with 0 being true black and 255 true white. Subsequently, we standardized each image for white balance by adjusting the brightness in each separate photo from the image-specific greyscale value obtained from the black and white colour standard. Thereafter, body darkness was quantified as the percentage of dark pixels in three different body regions, central area (a1, 6×6 mm), dorsal area (a2, 6×6 mm) and ventral area (a3, 6×3 mm) (Fig. S1). This was done by adjusting each image for dark and white threshold values along the grey scale. Threshold values were decided upon initial observations and chosen to reduce extreme values, i.e. 0 and 100% dark pixels. For example, crucian carp are darker dorsally than ventrally, and, hence, we adjusted the threshold values for dark pixels specifically for each region accordingly: a1=30, a2=20 and a3=110, meaning that within a1, for example, values between 0 and 30 were considered 'dark', whereas values above 30 were considered 'light'.

Skin darkness and chromatophore analysis

To investigate the underlying proximate mechanisms behind potential treatment differences in body coloration, a subsample of fish from the four different treatment groups was haphazardly chosen. These fish ($n=86$) were frozen (−20°C) and subsequently sampled for chromatophore analyses using skin scales. In total, we collected 3–7 scales from the anterolateral part, just behind the operculum (i.e. within the a1 region; see Fig. S1). Scales were stored at 4°C in phosphate-buffered saline with 4% paraformaldehyde as a fixative until analysed. While fish can possess several different types of skin chromatophores (Fujii, 2000), black–brown melanophores (melanin) were found to be the most common in crucian carp skin, and we thus focused on these to quantify body darkness. Melanophore cell density, as a measure of morphological colour change, was scored manually by counting the number of melanophores in a 1 mm row perpendicular to the scale edge; see Fig. S2). Intracellular distribution of melanophore pigment, as a measure of physiological colour change, was scored manually under light microscopy using the melanophore index (MI) (Hogben and Slome, 1931) where MI=1 means that pigment is aggregated in the centre of each cell whereas MI=5 means that pigment is evenly distributed throughout each cell (see Fig. S3). A minimum of three different scales were analysed from each fish and mean MI and density values from each fish were used for statistical comparisons. Scoring and photography were performed using a light microscope equipped with a camera (Leica Microsystems AB, Kista, Sweden). Chromatophore status was assessed (by H.N.S.) blind with respect to sample identity and experimental treatment.

Statistical analyses

Relative body depth at the end of the experiment was analysed with ANCOVA, using individual body depth as dependent variable, treatment as factor and standard length as a covariate, including the standard length×treatment interaction term, followed by Fisher's

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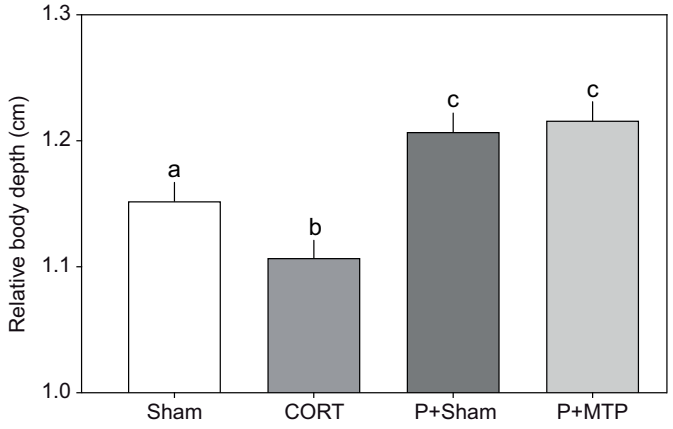

Fig. 1. Predicted mean relative body depth for fish from the four different treatments. Sham: predator absent with sham implant (n=35); CORT: predator absent with cortisol implant (n=28); P+Sham: predator present with sham implant (n=31); and P+MTP: predator present with metyrapone implant (a cortisol inhibitor; n=30). The means were calculated from the ANCOVA model and estimated for a fish of a fixed standard length (7.16 cm); whiskers indicate s.e.m. and different letters denote statistically significant differences between treatments.

LSD *post hoc* test. Body darkness, measured as the percentage of dark pixels in the three different body regions (a1, a2 and a3), was analysed with MANOVA and subsequent evaluation of univariate between-subject effects for significant MANOVA effects, followed by a pairwise comparison between treatments (Fisher's LSD based on the estimated marginal means). The chromatophore analyses of scale samples were tested in a separate MANOVA because of the reduced sample size of this subsample. In all models, we nested tank identity within treatment. The residuals from each model were tested for normality and all data met the assumption of normal distribution. All statistical analyses were performed using SPSS v.23.0 for Mac OS X (SPSS Inc., Chicago, IL, USA).

RESULTS

Inducible morphological defence

The full ANCOVA revealed a non-significant standard length×treatment interaction term ($F_{3,96.00}$=2.06, P=0.111), and the model was reduced and run without the interaction term. The relative body depth of individual fish was strongly influenced by treatment (ANCOVA: $F_{3,30.27}$=11.06, P≤0.001). The *post hoc* analysis revealed that P+Sham fish expressed a deeper body shape compared with Sham (predator-free) fish (P=0.018). However, the relative body depth of fish from the CORT treatment was significantly more shallow compared with that of Sham fish (P=0.023). No effect of metyrapone was found on relative body depth, i.e. P+MTP fish did not differ from P+Sham fish (P=0.619; Fig. 1; for details, see Table S1).

Colour – image analysis

Treatment had a strong overall effect on body coloration (MANOVA, Wilks' λ: $F_{9,238.66}$=27.43, P<0.001; Fig. 2); furthermore, all areas analysed differed significantly across treatments (univariate between-subject effects for a1–a3: P<0.001). *Post hoc* test (LSD) revealed that CORT fish had a reduced percentage of dark pixels in regions a1 (P=0.011) and a2 (P=0.015) relative to Sham fish, whereas the two predator-exposed groups had a significantly higher percentage of dark pixels overall compared with Sham fish reared without a predatory pike (a1–a3, P<0.001; see Fig. 2). We found no effect of metyrapone on body coloration, i.e. P+MTP fish did not differ statistically in percentage of dark pixels compared with P+Sham fish (a1–a3, P>0.05; see Table S2 for more details).

Colour – chromatophore analysis

Both melanophore cell density and MI were significantly different between treatments (MANOVA, Wilks' λ: $F_{6,124.00}$=8.33, P≤0.001, Fig. 3). The univariate between-subject effects test revealed a strong treatment effect on melanophore cell density ($F_{3,63.00}$=15.40, P≤0.001) and a marginally (non-) significant treatment effect on MI ($F_{3,63.00}$=2.67, P=0.055). Specifically, crucian carp reared in the presence of a pike (P+Sham and P+MTP) had a significantly higher melanophore cell density than experimental fish reared in the absence of a predator (Sham and CORT), whereas no effects were found for either cortisol or metyrapone (Fig. 3A; Table S3C). The marginally (non-) significant treatment effect on MI is probably a trend arising from the high MI observed in Sham fish compared with a relatively low MI observed among CORT fish (P=0.008; Fig. 3B;

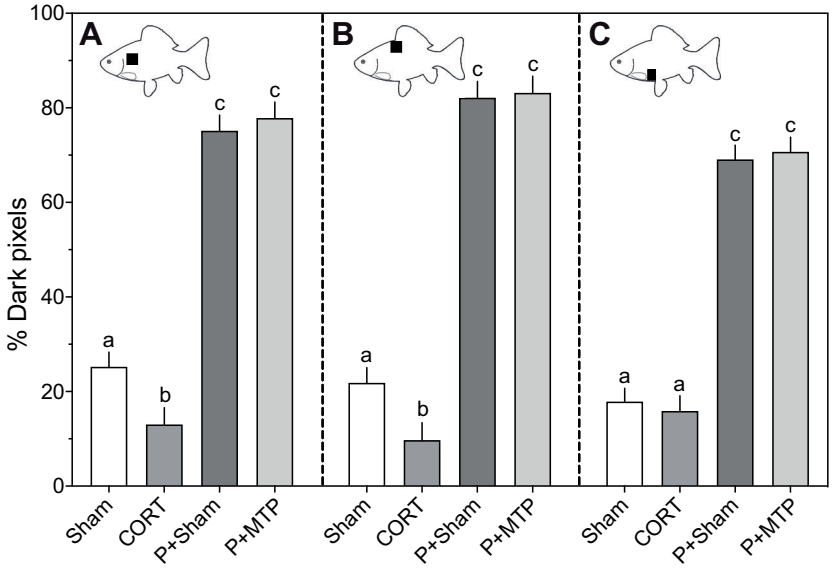

Fig. 2. Percentage of dark pixels within different areas of fish from the four treatment groups. Data are shown for (A) central (a1), (B) dorsal (a2) and (C) ventral (a3) areas of the fish (Sham, n=35; CORT, n=28; P+Sham, n=31; and P+MTP, n=30). Means±s.e.m.; different letters denote statistically significant differences between treatments.

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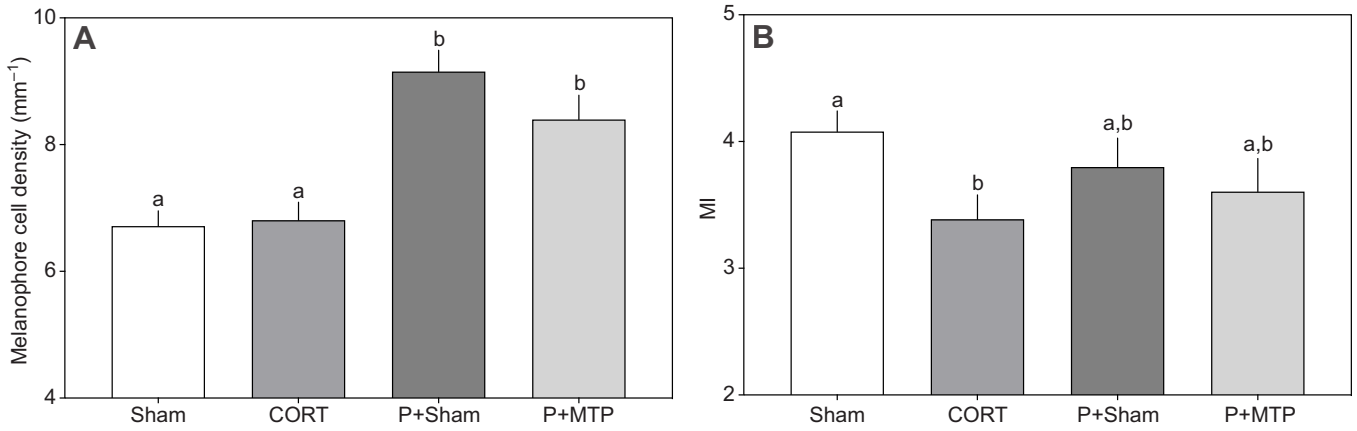

Fig. 3. Melanophore cell density and index (MI) of fish from the four treatment groups. (A) Melanophore cell density and (B) MI (Sham, *n*=29; CORT, *n*=22; P+Sham, *n*=20; and P+MTP, *n*=15). MI=1 means that pigment is aggregated in the centre of each cell whereas MI=5 means that pigment is evenly distributed throughout each cell, leading to lighter and darker appearance, respectively. Means±s.e.m.; different letters denote statistically significant differences between treatments.

Table S3). Hence, no effect of predation risk was found on MI (see Table S3).

DISCUSSION

We found that crucian carp show pronounced plasticity in both colour and body-shape traits in response to perceived predation risk and to manipulation of a main glucocorticoid (cortisol) via intraperitoneal implants. Crucian carp exposed to predatory pike expressed a darker coloration and developed a deeper body morphology. Furthermore, we report that treatment with implants containing cortisol resulted in lighter body coloration and shallower body depth as compared with those of Sham fish also reared in the absence of a predator. Intriguingly, implants containing cortisol thus elicited a trajectory of trait changes contrasting with the phenotype expressed under direct predator exposure.

The ability to change body colour is a striking and obvious example of phenotypic plasticity. It is a strategy particularly prevalent among aquatic and semi-aquatic species, such as cephalopods (Messenger, 2001), reptiles (Lewis et al., 2017; Stuart-Fox and Moussalli, 2009), amphibians (McCollum and Leimberger, 1997; Touchon and Warkentin, 2008) and fish (Rodgers et al., 2010; Ryer et al., 2008). Many teleost fish have evolved the capability to alter their body coloration to match the environmental background (Cott, 1940), enabling predatory fish to attack via ambush, or prey to enjoy enhanced survival chances from crypsis (Leclercq et al., 2010; Rodgers et al., 2010; Ryer et al., 2008; Sumner, 1935). However, to our knowledge, the darkening of body coloration as observed among crucian carp exposed to predatory pike is the first example of a teleost colour change directly induced by controlled manipulation of the predator environment. In addition, we were also able to show that the colour change in predator-exposed crucian carp is mediated by a higher melanophore cell density, whereas no effect of predation risk was found in pigment dispersion within the melanophores (MI). Our findings thus show that it is melanogenesis, i.e. a morphological colour change, that underlies the induced darkening of the body in predator-exposed crucian carp (Leclercq et al., 2010; Sugimoto, 2002). Such colour change takes a substantially longer time, and is often induced by adverse conditions where the enrolment of the stress axis is considered to be the key factor behind the increased melanin biosynthesis (Leclercq et al., 2010). The adaptive value of the colour change in crucian carp may be an accentuation of the predator-induced morphological defence. Hence, individuals that have invested in the morphological defence may benefit from displaying their deep, defended phenotype via a dark and distinct silhouette to selective predators such as pike, known to avoid such phenotypes (Nilsson et al., 1995). Hence, a darker body may act as an effective aposematic signal (Caro, 2009; Rojas et al., 2015). However, the presence of pike also induced shifts in diel activity patterns towards nocturnality in crucian carp (J.V., K.H., D. E. Nilsson, P.A.N. and C.B., unpublished data). Under such conditions, a darker body may also contribute to crypsis and reduce the risk of detection by visually oriented predators, such as pike.

In contrast, fish that received a cortisol implant had a more shallow relative body depth and a lighter body coloration as compared with Sham fish. These findings were not in line with our initial prediction and do not align with a previous experiment on amphibian tadpoles where experimental exposure to corticosterone elicited trait changes analogous to the predator-induced phenotype (Maher et al., 2013). The opposite effects of glucocorticoids on predator-induced traits in amphibian tadpoles and crucian carp suggest different evolutionary pathways for the physiological mechanisms that regulate inducible defence expressions. Yet, in the light of the complex endocrinological pathway of the vertebrate stress axis, our results suggest that the HPI axis may also have a role in the regulation of plastic defence expression in our model system.

Numerous studies have examined the proximate and ultimate functions of the stress axis in prey upon predator exposure (e.g. Giesing et al., 2011; Hammerschlag et al., 2017; Oliveira et al., 2014; Sapolsky et al., 2000). The stress response is self-regulated via a negative feedback system where the majority of steroid actions, such as glucocorticoid production and secretion, are under genetic control from the proopiomelanocortin (*POMC*) gene. Translation of *POMC* produces a precursor resulting in multiple peptides, including hormones having various phenotypic effects (Harris et al., 2014; Navarro et al., 2016). Among these *POMC*-derived peptides, the so-called melanocortins (i) adrenocorticotropic hormone (ACTH) and (ii) α-melanophore-stimulating hormone (α-MSH) are of particular interest as they are involved in both stress physiology and pigment production. Earlier studies have demonstrated their principal roles in the process of melanogenesis and pigment dispersion/aggregation (Cal et al., 2017; Cerdá-Reverter et al., 2011; Ducrest et al., 2008; Fujii, 2000; Leclercq et al., 2010; Sköld et al., 2015), as well as their role as main

regulatory agents in the vertebrate stress axis (Aguilera, 1994; Sapolsky et al., 2000). For example, ACTH stimulates the adrenal/interrenal gland to produce and secrete cortisol (Slominski et al., 2000; Sumpter et al., 1986). Further, enhanced glucocorticoid concentrations subsequently suppress the pituitary expression of *POMC*, resulting in reduced levels of ACTH and α-MSH (Aguilera, 1994; Drouin et al., 1989; Slominski et al., 2000). This self-regulation of the stress axis is important to regain homeostasis after an acute stressor and to avoid lethal effects from high glucocorticoid levels. Chronically stressful environments, as experienced by prey in the presence of persistent predator cues, may cause sustained physiological stress with glucocorticoid concentrations above baseline levels (Balm and Pottinger, 1995; Boonstra, 2013; Clinchy et al., 2013; Hammerschlag et al., 2017; Maher et al., 2013).

The interaction between the melanocortin system and vertebrate stress physiology creates an intriguing link behind our treatment-induced trait alterations. In salmonids, for example, the presence of dominant individuals has been shown to alter the body coloration among subordinate conspecifics towards a darker body colour. Proximately, this was caused by higher levels of ACTH and α-MSH from enhanced stress levels in the subordinate individuals, i.e. stress-induced melanogenesis (Höglund et al., 2000). The same underlying mechanism could explain the morphological colour change that we observed among predator-exposed crucian carp, although, from our experiment, it is not possible to determine whether the treatments altered the activity rates of the HPI axis differently. However, it should be expected that the constant presence of a pike predator, involving both visual and chemical cues, caused an elevated stress level and thereby enhanced melanogenesis from higher transcription and translation of the *POMC* gene (Boonstra, 2013; Clinchy et al., 2013; Hammerschlag et al., 2017; Hossie et al., 2010; Maher et al., 2013). Further, and vice versa, intraperitoneal delivery of cortisol should suppress the expression of *POMC* via a negative feedback loop, resulting in a reduced endocrine stress response and reduced melanogenesis/pigment dispersion within the melanophores (Cal et al., 2017; Fujii, 2000; Leclercq et al., 2010; Sköld et al., 2015). Implants containing cortisol have indeed been shown to successfully suppress the HPI axis in goldfish (*Carassius auratus*) (Bernier et al., 1999; Fryer and Peter, 1977), a species closely related to crucian carp. However, cortisol did not increase the melanophore cell density as predator exposure did; instead, we found that fish originating from the CORT group had lower MI compared with Sham fish. Lower MI is unambiguously the mechanistic explanation for why CORT fish had a lighter body coloration in the central and dorsal region compared with Sham fish. ACTH and α-MSH drive melanin production (morphological colour change), but they also regulate pigment dispersion within the melanophores, leading to altered body darkness via a physiological colour change (Cal et al., 2017; Fujii, 2000; Sköld et al., 2015). This fits well with our earlier arguments, i.e. that implants containing cortisol may reduce melanocortin concentrations from the negative feedback suppression of *POMC* by free cortisol levels (e.g. Sapolsky et al., 2000), which in theory could explain the lower MI and lighter external appearance of CORT fish.

Steroid hormones, such as cortisol, may underlie phenotypic alteration in different traits. For example, experimental manipulation of cortisol via implants has been shown to influence growth rate (Bernier et al., 2004; Midwood et al., 2014), reproduction (Carragher et al., 1989; Crossin et al., 2016) and behaviour (Barreto et al., 2014). As earlier indicated (e.g. Höglund et al., 2000), we here add colour change to the growing literature of phenotypic alteration via cortisol implants. We found no effect of metyrapone, either on body coloration or on body shape. However, in contrast to cortisol, metyrapone has rarely been used with a cocoa butter carrier for prolonged distribution (>24 h) (Hopkins et al., 1995; Milligan, 2003; Rodela et al., 2012). In fact, one study revealed that the method of using cocoa butter as a vehicle for *in vivo* metyrapone delivery in salmonids only worked during the first days, and the effect was found to be absent 5 days post-injection (McConnachie et al., 2012). In comparison, numerous studies have found prolonged elevation (>5 weeks) of plasma cortisol from implants containing cortisol (Carragher et al., 1989; Pickering and Duston, 1983). These findings, and the lack of effect of metyrapone on the phenotypic trait expression in crucian carp, might be due to the short half-life of the drug per se, but it has also been suggested that metyrapone has different mechanisms of action across vertebrate groups. Doyon et al. (2006) found that metyrapone caused increased cortisol levels in rainbow trout (*Oncorhynchus mykiss*). Hence, the use of metyrapone for prolonged cortisol inhibition in fish is problematic, but our results indicate that cortisol could act as an inhibitor of the stress axis. This prediction is in line with earlier studies on teleost fish, where cortisol has been demonstrated to suppress the regulation of cortisol release (Bernier et al., 1999; Fryer et al., 1984; Fryer and Peter, 1977). Therefore, we argue that an experimental design where cortisol treatment is combined with predator presence would be an interesting next step as that combination of treatments may have the potential to disentangle the underlying physiological effects of melanocortins on the inducible morphological defences in crucian carp.

In conclusion, we show that crucian carp respond to predation risk by expressing a significantly darker body coloration, and that this is regulated by the melanocortin system, via enhanced melanogenesis. Such predator-induced morphological colour change might be adaptive, especially when integrated with the inducible morphological defence in this model system, i.e. a deeper body shape (Brönmark and Miner, 1992). A darker external appearance could constitute an enhanced silhouette from greater contrast of a deep-bodied and hard-to-capture prey phenotype and, hence, act as an aposematic signal to nearby predators (Caro, 2009; Rojas et al., 2015), or be a synchronized strategy for crypsis along with a nocturnal lifestyle (J.V., K.H., D. E. Nilsson, P.A.N. and C.B., unpublished data). However, colour change from increased melanization is energetically costly and might explain the brighter body coloration among crucian carp living in the absence of predatory pike (Rodgers et al., 2013). Given these results, predator prey-choice experiments are now needed to assess whether increased body coloration in combination with an increased body depth confers additive anti-predator effects, for example by displaying computerized animations of deep-bodied crucian carp with different simulated coloration to foraging pike (Ingley et al., 2015). Further, we have shown that cortisol alters the phenotypic expression of anti-predator traits, although in completely opposite trait trajectories from our predictions based on earlier work on amphibian tadpoles (Maher et al., 2013). Yet, we argue that our findings follow the theoretical prediction from a physiological perspective, i.e. that cortisol implants suppress the melanocortin system, leading to a significant effect on the individuals' coloration, and, intriguingly, influencing the body shape of fish by reducing relative body depth. We conclude that the endocrinological pathways of the vertebrate stress axis are of considerable interest when it comes to the interpretation of how different traits might be integrated, and for our understanding of the underlying mechanisms that regulate morphological defence expression in plastic organisms.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.V., K.H., P.A.N., H.N.S., C.B.; Methodology: J.V., K.H., P.A.N., H.N.S., C.B.; Validation: J.V., K.H., P.A.N., H.N.S., C.B.; Formal analysis: J.V., K.H., P.A.N., C.B.; Investigation: J.V., K.H., H.N.S.; Resources: H.N.S., C.B.; Data curation: J.V., H.N.S.; Writing - original draft: J.V., K.H.; Writing - review & editing: J.V., K.H., P.A.N., H.N.S., C.B.; Visualization: J.V.; Supervision: P.A.N., C.B.; Project administration: P.A.N., C.B.; Funding acquisition: C.B.

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Data availability
Data are available from the Dryad Digital Repository (Vinterstare et al., 2020): dryad.h70rxwdfr.

Supplementary information
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