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Sex matters: predator presence induces sexual dimorphism in a monomorphic prey, from stress genes to morphological defences

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Abstract

Inducible defences allow prey to increase survival chances when predators are present while avoiding unnecessary costs in their absence. Many studies report considerable inter-individual variation in inducible defence expression, yet what underlies this variation is poorly understood. A classic vertebrate example of a predator-induced morphological defence is the increased body depth in crucian carp (*Carassius carassius*), which reduces the risk of predation from gape-size limited predators. Here, we report that among-individual variation in morphological defence expression can be linked to sex. We documented sexual dimorphism in lakes in which crucian carp coexisted with predators, where females showed shallower relative body depths than males, but not in a predator-free lake. When exposing crucian carp from a population without predators to perceived predation risk in a laboratory environment (presence/absence of pike, *Esox lucius*), we found that males expressed significantly greater morphological defence than females, causing sexual dimorphism only in the presence of predators. We uncovered a correlative link between the sex-specific inducible phenotypic response and gene expression patterns in major stress-related genes (*POMC, MC3R,* and *MC4R*). Together, our results highlight that sex-specific responses may be an important, yet underappreciated, component underlying inter-individual differences in the expression of inducible defences, even in species without pronounced sexual dimorphism.

Keywords: phenotypic plasticity, stress physiology, inducible morphological defence, predator-prey interactions, *POMC*, crucian carp

Virtually all animals constitute potential prey; as such, they are under strong selection to avoid capture by their natural enemies. Consequently, a broad suite of anti-predator defences, involving striking phenotypic adaptions in behaviour, morphology, and physiology, are displayed in nature (Clinchy et al., 2013; Cott, 1940; Creel, 2018; Langerhans, 2007; Lima & Dill, 1990). There are multiple routes to efficient anti-predator defences, spanning a continuum from canalized (genetically fixed) to highly plastic trajectories of anti-predator trait change. At one end, constitutive defences are those always expressed by an organism, regardless of prevailing predation risk. However, prey can confront intermittent and unpredictable regimes of selection as predation risk often shows a high degree of spatio-temporal variability (Tollrian & Harvell, 1999). Such conditions may instead favor the evolution of phenotypic plasticity in anti-predator traits (e.g., Brönmark & Miner, 1992; McCollum & Leimberger, 1997; McCollum & Van Buskirk, 1996). Plasticity in defence traits (inducible defences) allows for flexible adjustment and fine-tuning of anti-predator traits in response to changes in predation pressure, increasing survival chances, and, thus, fitness (e.g., Cortesi et al., 2015; Nilsson et al., 1995). However, inducible defences are also expected to incur substantial costs (DeWitt et al., 1998), e.g., via a reduction in competitive ability (Pettersson & Brönmark, 1997), fecundity and growth (Brönmark et al., 2012), or infection tolerance (Yin et al., 2011) and, hence, no single phenotype is optimal in both high- and low-risk environments (McCollum & Van Buskirk, 1996). This phenotypic trade-off framework constitutes a key component of the modern evolutionary view of induced adaptive plasticity in defensive traits (DeWitt et al., 1998; Pigliucci, 2005; Tollrian & Harvell, 1999).

Numerous species, distributed across a wide range of taxa, adopt inducible defence strategies (Tollrian & Harvell, 1999), and these organisms have been extensively used as models for addressing the ecology and evolution of phenotypic plasticity (Hossie et al., 2010; Vinterstare et al., 2019; Weiss et al., 2015). Often there is considerable variability among individuals in the degree to which inducible defences are expressed (Ahlgren et al., 2015; Hulthén et al., 2014b; Meuthen et al., 2019). Here, a contemporary challenge is to move beyond describing this variability, and towards understanding the factors responsible for causing and maintaining such inter-individual variability (Mitchell et al., 2017) that may provide a substrate for selection and evolutionary processes behind inducible defences. A reason why individuals might vary in

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inducible defence trait expression can derive from sex-specific differences, as males and females can experience differences in the strength and direction of both natural and sexual selection. In a broad diversity of taxa, the sexes exhibit different anti-predator strategies, including sex-specific behaviors, morphologies, life histories, and physiological responses (e.g., Donelan & Trussell, 2020; Heinen-Kay et al., 2016; Riesch et al., 2020; Vinterstare et al., 2021). For instance, sexes often differ in susceptibility to predation (Christe et al., 2006; Donelan & Trussell, 2020; Hendry et al., 2006; Pocklington & Dill, 1995; Riesch et al., 2020; Sommer, 2000) owing to trait differences such as dimorphism in body size and shape (Hassell et al., 2012; Langerhans et al., 2007), ornamentation (Martin et al., 2014) or mating behaviors (Magurran & Seghers, 1994). Furthermore, the relative energetic investment in reproduction often differs strongly between the sexes (e.g., eggs are more costly than sperm) (Zera & Harshman, 2001), which may drive sex-specific trade-offs in the expression of costly anti-predator defences (Meuthen et al., 2018, 2019). Sex differences in energy storage, mitochondrial metabolism, hormonal profiles, and habitat use can also result in sex-specific selection on anti-predator strategies. It is thus perhaps not surprising that recent work has begun to uncover sex-specific inducible morphological defences, at least in species with pronounced sexual dimorphism (Meuthen et al., 2018; Stillwell et al., 2010; Välimäki et al., 2012). However, sex-specificity of inducible defences may also arise in species lacking overt dimorphism in non-genital morphological traits and, thus, more attention is needed to be paid to the role of sex in explaining inter-individual variation in inducible defences.

Contemporary studies suggest that inducible defence expression may be mediated by physiological stress-response mechanisms, specifically predator-induced activation of the hypothalamus-pituitary-adrenal/interrenal axis (HPA/HPI axis), resulting in enhanced levels of associated stress hormones (glucocorticoids) (Hossie et al., 2010; Maher et al., 2013; Vinterstare et al., 2020b). For example, changes in glucocorticoid concentrations, caused by either predator exposure or experimental corticosterone manipulation, was shown to trigger the expression of an adaptive inducible morphological defence in amphibian tadpoles (Maher et al., 2013). Changes in stress-related glucocorticoid release are largely driven by the release rate and absolute levels of adrenocorticotropic hormone (ACTH) and alpha-melanocyte-stimulating hormone (α-MSH). ACTH and α-MSH are directly derived from the polypeptide precursor pro-opiomelanocortin (POMC), their release is induced by corticotropin-releasing factors (CRFs), and they activate melanocortin receptors (MCRs) (Harno et al., 2018; Metz et al., 2005). Together, these three gene types (*CRF, POMC,* and *MCR*) form a core of genes whose expression plays important roles in stress physiology (Ducrest et al., 2008; Harno et al., 2018; Sapolsky et al., 2000) and in the melanocortin system, involving proximate effects on, e.g., pigmentation, appetite, and energy homeostasis (Cone, 2006; Gantz & Fong, 2003). Hence, these genes may be involved in the regulation of inducible morphological defences via risk-dependent glucocorticoid release (e.g., Maher et al., 2013). Intriguingly, recent studies have shown important sex-specific differences in the evolution of both the behavioral and physiological responses to stressors (Houslay et al., 2019; Rambo et al., 2017; Vinterstare et al., 2021). For example, earlier studies have examined sex-specific responses in stress physiology across environments varying in risk (Giesing et al., 2011; McGhee et al., 2020), and, how predator-induced changes in stress hormones influence, e.g., reproductive effort and outcome (Dulude-de Broin et al., 2020; Humphrey et al., 2020). These findings further suggest a proximate link from sex-specific variation in stress responses to evolved differences in inducible defence expression.

Perhaps due to logistical constraints, most research on sex-specific morphological plasticity has focused on species in which sex can be readily determined from external characteristics, such as sexually dimorphic ornamentation in poeciliids, sticklebacks, and cichlid fish (Kotrschal et al., 2012; Meuthen et al., 2018, 2019; Välimäki et al., 2012). In many animals, however, including the majority of teleost fish species, sexual dimorphism is often either absent or relatively weak during most of the annual cycle (Ross, 1984; Wearmouth & Sims, 2008). Still, sexes may experience different selection on anti-predator phenotypes even in such monomorphic species. Moreover, most studies aiming at shedding new light on sex-specific plasticity have gathered data either in the field or laboratory environment, which is unfortunate as changes from a natural to a captive environment can alter behavior and stress physiology, including baseline and stress-induced plasma glucocorticoid levels (Marra et al., 1995). Alternatively, populations maintained in the artificial laboratory environment over multiple generations may have lost plasticity (Morgan et al., 2022). This highlights the need to evaluate hypotheses regarding sex-specific plasticity in anti-predator defences under both natural and controlled laboratory conditions. Such studies may allow for better inference about whether observed patterns and effect sizes are biologically relevant in natural environments, where multiple biotic and abiotic factors can exert force on phenotypic plasticity. Finally, although inducible defences are relatively well studied at the phenomenological level, the proximate basis of inter-individual variation in the magnitude of inducible defence expression, such as sex-specific gene expression, remains elusive. Recent studies have started to explore the intriguing amount of individual variation in defence expression (Hulthén et al., 2014b; Meuthen et al., 2019), but there is still a critical gap in our knowledge on the proximate, physiological mechanisms that regulate the expression of inducible defences, driving intra-individual variation.

Crucian carp (*Carassius carassius*) exhibit a striking inducible morphological defence: when exposed to chemical cues released by predators, such as pike (*Esox lucius*), they increase in body depth (Brönmark & Miner, 1992; Brönmark & Pettersson, 1994). The morphologically defended phenotype constitutes less desirable prey for gape-limited predators (Nilsson et al., 1995), and the deep body improves escape performance via enhanced locomotor capacity (Domenici et al., 2008). Previous research suggests that predator-induced phenotypic plasticity may be partly mediated the hypothalamic–pituitary–interrenal (*HPI*) axis in crucian carp (Vinterstare et al., 2020b), which opens up for the possibility that the expression of major stress genes may be involved in inducible defence regulation in this species. Here, we take an integrative approach to (1) assess whether sex-specific variation may underlie inter-individual variation in inducible defence expression, in the wild as well as in controlled laboratory experiments, and (2) assess whether expression of candidate stress genes might provide a proximate mechanism for any sex differences in inducible morphological anti-predator defence.

Organisms operate on a finite energy reserve, and, hence, because limited resources may result in an energetic trade-off between allocation toward reproduction vs. inducible morphological defences, we predicted that the well-established disparity in the energetic cost of reproduction between the sexes should result in females having less resources available to allocate towards the expression of defences. To test this hypothesis, we first asked whether standing variation in relative body depth present in two lake populations could be explained by sex differences. Next, we quantified sex-specific predator-induced changes in body depth at the level of individuals in previously predator-naïve crucian carp following experimental manipulation of perceived predation risk in a controlled laboratory environment. Following the observation of sex-specific differences in predator-induced morphological plasticity (see Results), we also took an important first step toward understanding the proximate genetic/physiological mechanisms underlying inter-individual variation in inducible defences by examining the expression profiles of key candidate genes in brain and kidney samples from a subset of the laboratory-reared individuals of both sexes originating from either control or predator treatments. Specifically, we investigated the important stress genes/gene families of *CRF, POMC,* and *MCR* due to their importance in the HPI axis and in the melanocortin system (Cone, 2006; Ducrest et al., 2008; Gantz & Fong, 2003; Harno et al., 2018; Metz et al., 2005; Sapolsky et al., 2000).

Materials and methods

Field study

Crucian carp were caught from two lakes in southern Sweden: lakes Bergundasjön (56°50′55.6″N 14°46′51.5″E) and Häckebergasjön (55°34′32.2″N 13°25′28.2″E). These lakes hold a number of different piscivorous fish species, such as pike (*E. lucius*), Eurasian perch (*Perca fluviatilis*) and European eel (*Anguilla anguilla*). Crucian carp occur at very low densities in lakes with piscivores (Brönmark et al., 1995; Holopainen et al., 1997b), and, hence, to obtain an adequate sample size we employed multiple (12–20) large-sized (leader 45–100 m and 20–45 m^2 catch area) fyke nets to fish during several weeks. In total, 366 individuals were caught in the spring of 2017 (Bergundasjön, *n* tot = 200, *n* males = 115, *n* females = 85, standard length: 30.24 ± 0.21cm; mean ± SE) and 2018 (Häckebergasjön, *n* tot = 166, *n* males = 75, *n* females = 91, standard length: 27.39 ± 0.39 cm). Fish were transported to the laboratory facilities at Lund University where we measured the body mass (nearest 0.1 g), determined the sex from gonad examination and took photographs of each specimen (lateral view) for morphometric analyses.

Laboratory experiment

Crucian carp were caught from a predator-free lake (size: 3.7 ha, location: 55°46.386′N 13°28.811′E) between the 23rd of May and 6th of June 2016. Collecting fish from a site lacking predators allowed us to assess if sexual dimorphism was evident in a natural population under a low-risk scenario at baseline (prior to experimental manipulation of predation risk), but also allowed us to track sex-specific morphological changes in response to elevated predation risk. Fish were caught with a fyke net and immediately transported to Lund University. We individually tagged each fish on the 8th of June 2016 by surgically implanting PIT-tags (Passive Integrated Transponder, HDX, Oregon RFID, size: 12.0 mm long, 2.1 mm diameter, weight in air 0.1 g) into the abdominal cavity, following Skov et al. (2005) and (Hulthén et al. 2014a), to enable individual tracking over the experimental period. In addition, all fish (*n* = 70) used in the current study acted as controls in a different experiment, which required an additional implant into the stomach cavity (silicone tube containing plain cocoa butter). Prior to experimentation, all fish were photographed laterally (see details below) for morphological assessment. These pre-treatment images were taken to ensure no morphological differences existed between treatments by chance prior to experimentation, but also allowed us to assess the degree of standing sex-specific morphological variation in a natural population inhabiting a predator-free environment. Implants and PIT-tags could potentially trigger sex-specific responses, that may in turn impact defence expression. However, such a scenario is unlikely to confound our interpretations given that all fish were treated in the same way, and no evidence for any such effects is known (Hulthén et al., 2014a, 2014b). Next, fish were distributed into experimental aquaria (152 L; 95 × 40 × 40 cm, five fish per aquarium) and randomly assigned to one of the two treatments (predator presence or absence), seven replicate tanks per treatment. Aquaria were filled with aerated tap water that was continuously filtered through a 10 cm thick 10 PPI foam filter. Each aquarium was divided into two halves by a perforated, transparent acrylic glass partition, resulting in two equally sized compartments, one housing crucian carp and the other housing a predatory pike (predator treatments) or left empty (controls). This set-up allowed experimental fish in the predator treatment to experience both visual and chemical cues from the pike. We also introduced artificial structure (a plastic macrophyte imitation, 20 cm), and prevented visual interaction between replicate tanks by attaching a blue opaque plastic film to three outer surfaces of each aquarium. Crucian carp were fed a mixture of frozen *Daphnia* and chironomids five times weekly at a ratio of approximately 5% of the total body mass in each replicate tank. Fish that died during the experiment (*n* = 8) were replaced with similar-sized fish to maintain constant density in all replicates throughout the experimental period. Further, when terminating the experiment, we found (via dissection) that 3 fish were infected by the parasitic tapeworm *Ligula intestinalis*. These 11 individuals (reserves + infected) were not included in any analyses. Predatory pike (size range: 24–31 cm) were caught by electrofishing in lake Krankesjön, southern Sweden, between the 15th and 17th of June 2016, and acclimatized to the lab environment in five 500–700L aerated holding tanks that contained artificial vegetation. The acclimatization period lasted for at least five days prior to the experimental start. Pike were fed a strict crucian carp diet (one crucian carp at one or two occasions per week and always with the same feeding rate in all experimental aquaria) throughout the experiment, and were introduced into the experimental aquaria on the 22nd and 23rd of June, i.e., when the experimental period was initiated.

Morphology of experimental animals

After six months (181 days), all crucian carp were removed from the experimental aquaria, anaesthetized with benzocaine (Sigma Aldrich, Ethyl p-Aminobenzoate), placed laterally on a white foam board, and digitally photographed. We used a digital single lens reflex (DSLR), Canon EOS 80D (Canon Inc., Tokyo) equipped with a 18–35 mm lens (f/1,8

DC HSM, Sigma Inc., Kawasaki). The camera was vertically mounted on a copy stand, connected to a desktop computer, and images (resolution: 6000 × 4000 pixels) were captured (at 35 mm focal length) using the live-view function of the DSLR and the EOS Utility 3 software (Canon inc.). A ruler was included in each image for scale calibration. After photography, all fish were dissected, and gonads were used to determine sex. In total, 35 males and 24 females were included in the experiment (17 males and 15 females exposed to predators, 18 males and 9 females held under control conditions). Both sexes were present within every tank but one, with an average of 1.9 females and 2.5 males in each tank.

From the images, we first measured standard length (SL) as the distance between the tip of the snout to the end of the last scale anterior to the caudal fin. We used landmark-based geometric morphometrics to quantify morphological variation. In total, we digitized 11 homologous landmarks on each fish (see Supplementary Figure S1) using tpsDig2 (Rohlf, 2017). With these 11 landmarks from all 425 fish (field study: n = 366, laboratory experiment: n = 59), we performed Generalized Procrustes Analysis (GPA) to scale, rotate, and superimpose landmarks (removing isometric size effects and all other non-shape variation). We performed a single GPA for all specimens (field and lab) so we could visualize all fish along the same morphological axes. Subsequently, we used tpsRelw (Rohlf, 2019) to extract centroid size (the square root of the sum of squared distances from landmarks to their centroid) as an estimate of body size and to perform Relative Warps Analysis (RWA; a Principal Component Analysis of the geometric shape data) to generate geometric morphometric descriptors of body shape. We retained the first seven RW axes for further analysis, explaining 91.2% of shape variation (Supplementary Table S1). In order to retain statistical power (especially for the laboratory experiment), we focused on these seven RWs to test for shape differences between lakes, sexes, and laboratory treatments.

We further performed two additional analyses using geometric morphometric data for field and laboratory fish: (1) we performed separate GPAs and RWs for field (Supplementary Table S2) and laboratory (Supplementary Table S4) specimens, respectively, and (2) to ensure no difference in morphology among replicates prior to the start of the experiment we performed a GPA using data from photographs taken both before and after experimentation (Supplementary Tables S3 and S4). Results of these analyses confirm the robustness of the results presented here (Supplementary Tables S2–S4). Because defence expression in crucian carp has often been quantified as the ratio between either the total body depth or the lateral line body depth and SL (Brönmark & Miner, 1992; Hulthén et al., 2014b; Vinterstare et al., 2019), we also present results of linear measurements in the supplementary material (see Supplementary Figure S3) to enable direct comparison with earlier studies.

Statistical analyses

We tested for sexual dimorphism in body shape of fish collected in the wild by conducting a multivariate analysis of covariance (MANCOVA) with the seven RW axis scores as the dependent variables and lake, sex, and their interaction as independent variables. We included centroid size as a covariate to control for allometry, and additionally tested for heterogeneity of slopes (interactions with centroid size). We were primarily interested in differences between sexes (the sex term), and whether sex-specific differences were consistent or varied between the two lakes (interaction between sex and lake). To assess the nature of shape variation responsible for any important model terms, we conducted follow-up univariate analyses to determine which RW axes were responsible for the effects and visualized relevant axes using thin-plate spline transformation grids. To provide an estimate of effect size and quantify the magnitude of body shape differences between sexes directly comparable to other studies, we calculated the average Procrustes distance between sexes within each lake using tpsSmall (Rohlf, 2017). Procrustes distance represents the standard metric of shape differences in geometric morphometrics (e.g., Bookstein, 1996) and is closely approximated by Euclidean distance between landmarks after GPA (Zelditch et al., 2012).

For fish from the laboratory experiment, we tested for predator-induced changes in body shape, and their sex dependence, by conducting a mixed-model nested MANCOVA (see e.g., Hassell et al., 2012; Riesch et al., 2013) using the seven RW axis scores as dependent variables, centroid size as a covariate, predator exposure treatment, sex, and the interaction between predator exposure treatment and sex as independent variables, and tank nested within predator treatment as a random effect. We confirmed homogeneity of slopes (all interactions with centroid size $p > .17$). p values were calculated using maximum likelihood and the Kenward-Roger degrees of freedom adjustment (see sample code in Riesch et al. (2013)). In this experiment, we were especially interested in the interaction between sex and predator exposure, as this term tests the hypothesis that sexes differ in their predator-induced morphological defence. We again conducted follow-up univariate tests to determine which RW axes were most responsible for the observed effects, and visualized shape variation using thin-plate spline transformation grids. We tested for relevant group differences using post-hoc Tukey's tests. Using tpsSmall, we calculated average Procrustes distance between treatments for each sex, and between sexes within each treatment. Morphological analyses were conducted using SAS software (version 9.3, SAS Institute, Cary, NC, United States).

Tissue sampling and sex determination

The experiment was terminated the day after all fish were photographed (in total 182 days of treatment exposure), when we first sacrificed one individual per replicate for tissue sampling for subsequent gene expression analysis, and, second, we also sacrificed all remaining individuals in order to determine sex of all individuals by dissection and inspection of each individual's gonads. In brief, fish were netted from their tanks and immediately euthanized with an overdose of benzocaine (Sigma Aldrich, Ethyl p-Aminobenzoate). Subsequently, brains (excluding *bulbus olfactorius*) and kidneys were dissected and placed in Eppendorf tubes containing RNAlater (Qiagen). We examined expression of our candidate genes within both the brain and kidney because these genes are known to show expression in one or both of these regions, with the brain and kidney representing the two primary locations of action for the HPI axis and melanocortin system of teleost fishes (Kobayashi et al., 2011; Metz et al., 2005; Mommsen et al., 1999). All samples were stored in a −80 °C freezer until RNA extraction. In the present study, we used whole-brain and whole-kidney samples following the protocols of earlier studies in teleosts (see e.g., Pavlidis

et al., 2015; Valen et al., 2011; Zhang et al., 2015, 2019). However, the *bulbus olfactorius* had to be excluded from all brain samples due to constraints with the dissection method employed.

RNA extraction

Individual samples were thawed and then transferred to a piece of aluminum foil and cut using sterile scalpels and forceps. Samples were cut so that the weight ranged between 20 and 30 mg, i.e., within protocol recommendation and done to avoid overloading of the spin columns while assuring sufficient yields of RNA (Qiagen RNeasy Plus Universal Kit). All samples were disrupted and homogenized by first being individually placed in 900 µl of QIAzol Lysis Reagent and subsequently in a Qiagen TissueLyser (Retsch). Thereafter, we used the Qiagen RNeasy Plus Universal Handbook (12/2014) protocol, with the optional step of an extra spin to eliminate any possible carryover of RPE buffer. The samples (n tot = 14, n predator-exposed = 7; n male = 4, n = female = 3, n predator-free = 7; n male = 4, n female = 3) were eluated in 50 µl RNase-free water and analyzed for quantity and quality on an Agilent 2100 BioAnalyzer.

Gene expression

All the raw reads obtained from RNA sequencing was quality trimmed by removing the adapters, low quality bases (Q < 20) and reads smaller than 20 bps from the dataset using Nesoni clip. All the clean reads (871 GB) obtained after quality trimming were used to construct a *de novo* assembly using Trinity version 2.4.0 at default parameters (Grabherr et al., 2011). The initial *de novo* assembly obtained from Trinity was subjected to subsequent quality filtering steps. First, TransDecoder version 5.3 (Haas et al., 2013) was used to identify the candidate coding region in the assembled transcripts, then single best open reading frame (ORF) per transcript was selected using TransDecoder—single_best_only. Transcripts with ORF less than 200 bps in length (potentially poor quality) were filtered out from the assembled transcriptome. Second, redundancy was removed from the assembled transcriptome by clustering highly similar transcripts using CD-Hit version 4.6.8 (Li & Godzik, 2006) at an amino acid sequence identity threshold of 1.00. All the high-quality transcripts obtained were used for further downstream analysis. BUSCO version 2.0.1 (Simão et al., 2015) was used to perform quality assessment of the assembled transcriptome using lineage "actinopterygii" by orthologous database odb9. The high-quality transcripts were annotated by BLASTP using NCBI non-redundant (nr) protein database at E-value cut-off of 1e-5. The protein domains were identified using InterProScan. For GO annotations and enzyme annotation by KEGG, the BLASTP, and InterProScan results were imported into BLAST2GO (Götz et al., 2008).

To specifically test the hypothesis that inducible defence expression is partly mediated by the HPI axis, we focused exclusively on expression of major genes involved in the physiological stress response and melanocortin system. We identified all copies of *CRF, POMC,* and *MCR* (including *MRAP*) genes within the data for analysis. For gene expression analysis of each gene, first abundance estimation was performed with RSEM version 1.3.1 (Li & Dewey, 2011) at default parameters followed by differential expression analysis with EdgeR (Robinson et al., 2009; McCarthy et al., 2012). Whenever we found multiple copies of a candidate gene (see Results), we analyzed all copies for differential gene expression.

To test for sex-specific responses in gene expression between predator treatments, we conducted generalized linear models that included sex, predator treatment, and their interaction as independent variables, and normalized gene expression values for each gene as the response variable. Each generalized linear model was fitted with either a Poisson or normal distribution based on the best-fitting distribution using AIC_c. In an effort to identify potentially differentially expressed genes for future study while adjusting for multiple comparisons—and in light of our moderate sample sizes—we chose to control for a false discovery rate (FDR) of 15% in our p values (Benjamini & Hochberg, 1995). *A priori*, we hypothesized that if expression of these genes influenced observed patterns of sexual dimorphism, then sex differences in gene expression should depend on predator treatment (Sex × Predator Treatment). Analyses were performed using JMP software (version 16, SAS Institute, Cary, NC, United States).

Results

Sex-specific variation in high-predation sites

We found a strong difference between the sexes in body mass, where female crucian carp were significantly heavier than males in both lake Bergundasjön and lake Häckebergasjön (See Supplementary Figure S3). For the two lake populations, both coexisting with predators, we found significant variation in body shape between lakes and between sexes, but no interaction between lake and sex (Table 1). We further observed a significant interaction term between lake and centroid size (p < .001), indicating that allometry differed between lakes, with a steeper decrease in body depth with increasing centroid size in lake Bergundasjön. Such allometric variation can complicate inferences regarding size-independent body shape, but we found this effect was quite weak relative to other effects, e.g., using Wilks' partial η^2 as an effect-size estimate, the effect of sex was approximately 3.7 times larger than the interaction-term effect. Moreover, inclusion of this interaction term had very little influence on interpretation of shape variation (e.g., correlation among least-squares means estimates for the seven RWs either including or excluding the term was r = 0.998). Thus, we can accurately interpret body shape variation among groups in the presence of this lake-specific allometry. Follow-up univariate tests showed that differences in body shape between sexes and lakes involved a number of RWs: sex effects were evident for RWs 1–3, 5; lake effects were evident for RWs 1–3, 6–7. Because RW1 explained over 61% of the shape variation, and because RW1 also served as the primary axis of variation relevant for

Table 1. Results from MANCOVA examining variation in body shape (7 RWs) of 366 field-collected crucian carp from two lake populations coexisting with predatory fish.

Term	F	df	p
Centroid size	29.03	7, 354	<.0001
Lake	170.49	7, 354	<.0001
Sex	21.76	7, 354	<.0001
Lake × Sex	1.63	7, 354	.1257
Lake × Centroid size	4.49	7, 354	<.0001

the laboratory experiment (see below), we visualized shape variation along this axis for both wild and laboratory specimens. We found that males had deeper bodies than females within each lake, while fish from lake Häckebergasjön had deeper bodies than those from lake Bergundasjön (Figure 1A). Average Procrustes distance between sexes within lake Bergundasjön was 0.0160 and within lake Häckebergasjön 0.0213.

Plastic defence regulation in the laboratory experiment

Importantly, the pre-treatment analyses failed to detect sexual dimorphism in the fish derived from a predator-free lake prior to being experimentally exposed to predator cues in the laboratory experiment (see Supplementary Material text and Table S3). At the conclusion of the experiment, we found effects of both predator treatment and the interaction term between predator treatment and sex on body-shape variation (Table 2). Follow-up univariate tests revealed that RW1 captured most of the relevant variation in body shape (strong effects of treatment and the interaction between treatment and sex), while only RW4 additionally showed variation among treatments, and no other RW exhibited variation associated with the interaction between treatment and sex. Examining Tukey's tests for RW1, we found that, while statistically controlling for body size, males induced deeper bodies when exposed to predator cues ($p < .0001$), while females did not ($p = .70$) (Figure 1B). This greater morphological defence response in males resulted in significant sexual dimorphism after exposure to predators (Tukey's test: $p = .017$), while fish held under predator-free conditions (control) remained

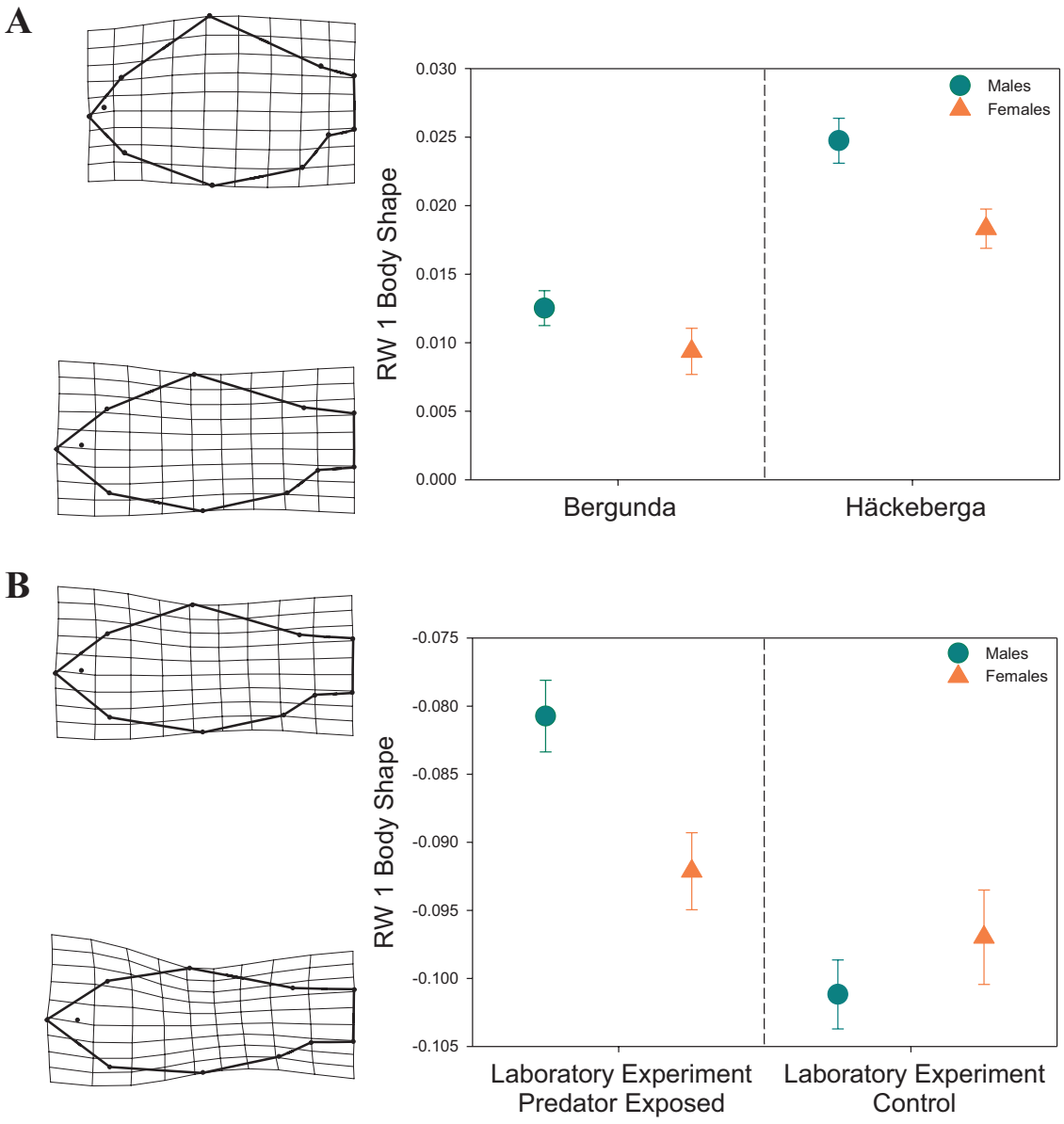

Figure 1. Variation in body shape among males and females (A) in two lake populations coexisting with predatory fishes and (B) when exposed/unexposed to predator cues in a laboratory experiment. Thin-plate spline transformation grids illustrate the observed ranges of body shapes relative to the overall mean, with lines drawn to aid interpretation of the body outline of the fish. Least-squares means ± SE depicted.

Table 2. Results from a mixed-model nested MANCOVA examining variation in body shape (7 RWs) of 59 crucian carp experimentally raised in either the presence or absence of predator cues. The model included tank identity as a random effect.

Term	*F*	*df*	*p*
Centroid Size	3.32	6, 172	.0041
Predator Treatment	6.63	6, 172	<.0001
Sex	0.66	6, 172	.6784
Predator Trt × Sex	2.68	6, 172	.0164

Table 3. *p* values from generalized linear models examining variation in expression of candidate genes in the brain and kidney. Bold indicates $p < 0.05$, * denotes significance after adjustment for a false discovery rate of 15%.

Gene	Brain			Kidney		
	Sex	Predator	Sex × Pred	Sex	Predator	Sex × Pred
POMC I	0.5934	0.3754	0.1670	**0.0207**	0.8842	**0.0206**
POMC II	0.4285	0.8106	**0.0108***	0.7191	0.7366	**0.0033***
POMC III	0.3685	0.0741	0.3145	—	—	—
MRAP2A	**0.0279**	0.8715	**0.0295**	0.2628	**0.0430**	0.1933
MRAP2B	0.8785	0.7897	0.6781	0.8787	0.7772	0.6600
MC3R	0.4149	0.3083	0.5637	0.2561	0.6589	**0.0011***
MC4R I	0.6605	0.2654	0.8732	—	—	—
MC4R II	0.9217	**0.0012***	**0.0077***	0.0822	0.9490	0.1663
MC5R I	0.7501	**0.0096***	0.6355	—	—	—
MC5R II	0.1761	0.2760	0.8517	—	—	—
CRF1	0.2278	0.5229	0.1226	0.7295	0.9626	0.0921
CRF2	0.8215	0.2678	0.2084	0.1155	0.1097	**0.0388**
CRF-BP	0.9392	0.4697	0.6768	0.0761	0.2781	0.7231

sexually monomorphic (Tukey's test: $p = .73$). In addition, we found significant allometry, i.e., larger fish had shallower bodies. Examining average Procrustes distances between groups mirrored these results, with more than twice as much body shape differentiation between treatments in males (0.0239) than females (0.0103), and much stronger differences in body shape between the sexes in the presence of predator cues (0.0151) than in their absence (0.0081). We found very little variation attributable to random tank effects (e.g., univariate Wald tests, all $p > .29$).

Gene expression profiles

In the assembled transcriptome, we identified multiple copies of several genes annotated as candidate genes of interest. Here, we refer to these copies with the suffixes *I*, *II*, or *III* (Supplementary Table S5). In four cases, all within the kidney (*POMC III, MC4R I, MC5R I,* and *MC5R II*), we found very low levels of gene expression and did not examine those cases further (average normalized expression of 0.08 vs. 7.95 for all others). After fitting the appropriate distribution to each generalized linear model (Supplementary Table S6), we found considerable evidence for sex-specific gene regulation in response to predators for our candidate genes (Table 3). Prior to FDR adjustment, we uncovered evidence for a significant interaction effect in 7 cases, spanning all the different types of genes (*CRF, POMC, MRAP,* and *MCR*) and both tissues. Four of these cases remained significant after our FDR adjustment, including two in the brain (*POMC II, MC4R II*) and two in the kidney (*POMC II, MC3R*), and all of them indicated greater expression in males than females only within the predator treatment (Figure 2). Generally, males tended to upregulate expression in these genes in the presence of predators (post-hoc tests $p \leq .05$ for all cases except *MC4R II* in brain), while females tended to show downregulation (post-hoc tests $p \leq .05$ for *MC3R* in kidney and *MC4R II* in brain, $p \leq .10$ for *POMC II* in brain and kidney). In addition to the observed interaction effects, we found two cases with evidence for overall differences between predator treatments, and one remained significant after FDR adjustment (Table 3). Specifically, both sexes exhibited greater expression of *MC5R I* in the brain in the presence of predator cues (Supplementary Figure S5). Meanwhile, we never observed clear evidence for sex differences in gene expression that was consistent across treatments. Details for the suggestive effects—those that did not remain statistically significant after FDR adjustment—are presented in the supplementary materials (Supplementary Figure S6).

Discussion

We found strong evidence for sex-specific differences at all three levels of investigation: body shape in the field, body shape in the laboratory experiment, and gene expression in the laboratory experiment. In the wild, male crucian carp in lakes with predatory fish had significantly deeper bodies than females—a pattern that was not observed in a predator-free lake and which could be caused by greater expression of the predator-induced morphological defence in males. In the laboratory experiment, adaptive morphological plasticity to predator cues was indeed only evident in males. This sex-specific inducible defence observed in the laboratory resulted in males being deeper bodied than females only in the presence of predatory fish, matching field observations. Differential expression of inducible defences could be caused by differential expression of key stress-physiology genes. Consistent with this notion, we found that for a number of candidate genes involved in the HPI axis, only males showed upregulation in the presence of predators. In line with previous work (Maher et al., 2013; Vinterstare et al., 2020b), we propose that our morphological and molecular results indicate a regulatory role of the stress axis and its neuroendocrine pathway involved in the evolution of predator-induced morphological defence.

Following our *a priori* prediction, results from the field clearly demonstrated that male crucian carp displayed a greater morphological defence phenotype as compared to females. Earlier studies focusing on sexually dimorphic traits in fish have shown that trait differences between sexes can be present during specific parts of the year, e.g., solely during the breeding season, or be constitutively expressed throughout the year (Smith & Wootton, 2016). However, sex-specific cost/benefit trade-offs in the presence and absence of predation risk can also drive the evolution of sexual dimorphism. For example, a recent examination of constitutively expressed morphological traits with anti-predator function among marine nine-spined sticklebacks demonstrated that males and females have evolved different strategies; males have deeper bodies whereas females display greater armor and longer dorsal spines (Välimäki et al., 2012). Further, predation has been shown to be a major force in driving the sexual dimorphism observed in several species of Poeciliidae (Hassell et al., 2012;

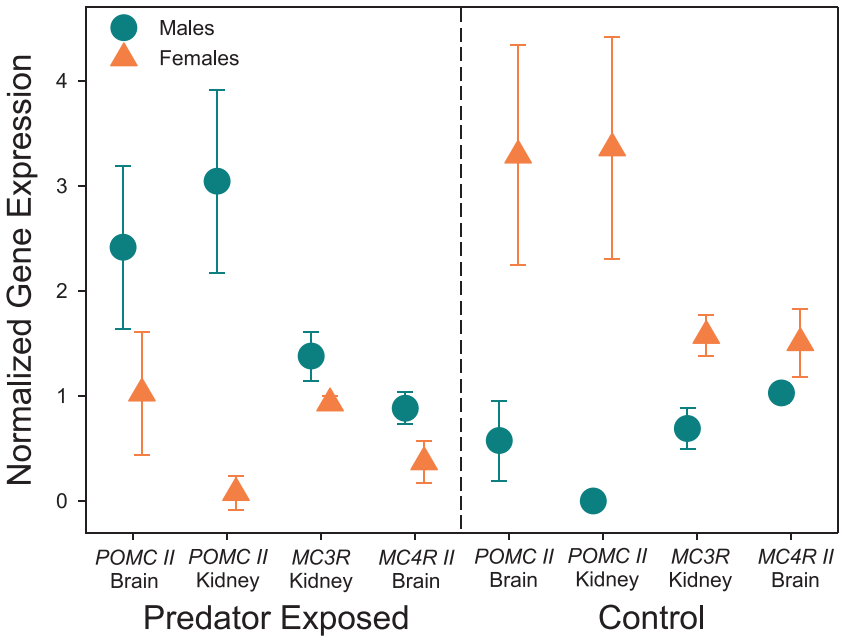

Figure 2. Expression of the four genes with strongest support for sex-specific differential expression across predator treatments. In the predator treatment, all of these genes showed greater expression in males than females, whereas the opposite pattern occurred in the control. Means ± SE depicted.

Langerhans & Makowicz, 2009). Hence, previous studies have mainly focused on examining sex differences in anti-predator strategies among organisms exhibiting overt dimorphism in morphological and/or behavioral traits. By focusing on crucian carp, a species that when occurring in low-risk environments display no known sex-specific trait disparity, we present novel insights into more cryptic sex-specific variation that only emerges under high-predation risk. The sexual dimorphism in the presence of predators was, when measured as Procrustes distance, about 0.015 in the laboratory experiment. That is similar to the magnitude of shape differences observed between habitat specialists in European whitefish (Siwertsson et al., 2013) and sympatric morphs of the Midas cichlid existing in a crater lake in Nicaruagua (Elmer et al., 2010). The Procrustes distances we observed among the sexes in lake Bergundasjön and within lake Häckebergasjön were, on average, even greater: Procrustes distances of 0.0160 and 0.0213, respectively.

Across species that lack pronounced sexual dimorphism, there is often sex-specific differences in the cost of reproduction, which may translate into differences in how prey behaviorally respond to and survive encounters with predators (Donelan & Trussell, 2020). Accordingly, female crucian carp have been shown to suppress chemically mediated fright responses during the spawning period (Lastein et al., 2008). Thus, in order to better understand the underlying causes for sex-specific differences in inducible defences, an obvious next step is to establish the degree to which more monomorphic species exhibit behavioral differences (e.g., sexual segregation in habitat choice and activity patterns) that may translate into sex-specific predation vulnerability in the wild.

In general, female gametes are much more expensive than male gametes, resulting in divergent resource allocation between the sexes into, e.g., reproduction and survival (Zera & Harshman, 2001). Such differentiated trade-offs, where females invest significantly more into reproduction compared to males, may explain our findings of a significantly dampened expression of the morphological defence (body depth) in females. Further, that females had a larger mass than males can potentially be explained by a sex-specific resource trade-off where females invest in general growth and enhanced fecundity and thus trade-off optimal shape for predator evasion, whereas males may gain higher lifetime fitness from investing into a more functional morphological defence against gape-size limited predators. Accordingly, an earlier field study compared the reproductive biology of female crucian carp originating from different habitats in the wild and demonstrated that both female GSI (Gonadosomatic index) and relative fecundity (eggs g^{-1} fish) are higher among individuals coexisting with piscivorous fish (see Holopainen et al., 1997b and references therein). Moreover, from earlier laboratory experiments, we know that female, but not male, crucian carp prioritize reproduction by suppressing the fright reaction during the final stages of sexual maturation (Lastein et al., 2008). This may further accentuate the general trade-off between reproductive effort vs. expressing defences. However, we lack data to disentangle the ultimate causation for why females express a dampened morphological defence against piscivorous predators compared to males. Although female gametes are relatively costly, males may also pay considerable reproductive costs, e.g., associated with the energetic costs of finding mates, male-male combat, and territory defence. It is also possible that females may experience different reproductive constraints on their body shape than males owing to their relatively large ovaries compared to the small testes of males (Hedrick & Temeles, 1989; Parker, 1992), which may partly explain our results.

The sex-specific differences in inducible defence expression we document here could further translate into differential survival among sexes in natural systems, resulting in skewed sex ratios. Natural populations of crucian carp inhabit lakes with and without piscivorous predators, but, unfortunately, we have no data on sex ratios in our focal lakes. However, data from other studies suggest an even or female-biased

sex-ratio in crucian carp in lakes without predators (de Meo et al., 2021; Tarkan et al., 2010), whereas in lakes with piscivorous fish, males were shown to occur at higher densities (up to 6.5 times) as compared to females (de Meo et al., 2021). Intriguingly, the skewedness in sex ratio changed along a gradient in predation pressure, with the most male-biased sex ratio in lakes with piscivore assemblages including the most efficient piscivore (pike), further suggesting the higher susceptibility of female crucian carp to predation due to a less expressed morphological defence. Large, adult females may enjoy a size refuge from gape-limited predation by all but the largest pike, but future studies should investigate age- and size-specific changes in skewedness in natural populations with and without piscivores.

Our second major result was in line with our initial prediction, and, further, parallel to the sex-specific shape differences observed in the field: male crucian carp displayed higher morphological plasticity by inducing deeper bodies than females in the presence of predators, matching the pattern observed in predator-exposed populations in the wild. Such inter-individual differences in plasticity and defence expression have previously been examined in this model system and linked to differences in personality types. Further, consistent differences in individual behavior, i.e., animal personality (Koolhaas et al., 2010; Reale et al., 2010; Sih et al., 2004), have been strongly associated with individual variation in the physiological response to different stressors (Koolhaas et al., 2010). Hence, physiology and changes in neuroendocrine pathways, as well as behavioral and morphological responses to predation threat, appear to be causally interrelated (Maher et al., 2013). For example, in live-bearing fishes, it has been demonstrated that males are bolder than female conspecifics (Harris et al., 2010; Heinen-Kay et al., 2016; Ingley et al., 2014), and male zebrafish show an increase in cortisol levels after a stressor whereas no increase in cortisol levels was found in stressed females (Rambo et al., 2017). How male and female crucian carp differ in their personalities is yet to be examined, but such sex-specific difference in personality traits may partly explain the findings of the personality-based regulation of morphological defence expression (Hulthén et al., 2014b).

The proximate mechanisms behind morphological plasticity are not well known, but recent studies suggests that the expression of inducible defence traits may be linked to changes in neuroendocrine pathways coupled to the physiological stress response (Hossie et al., 2010; Maher et al., 2013; Vinterstare et al., 2020b). Due to genetic variation, stress levels often differ between individuals, and have been shown to correlate with animal personality and coping styles towards diverse stressors (Fürtbauer et al., 2015; Koolhaas et al., 2010). The vertebrate stress axis is a complex machinery, involving a suite of hormonal, physiological, and behavioral responses. A key role of the vertebrate stress response is to prepare the individual for "fight or flight" through a neuroendocrine pathway resulting in enhanced glucocorticoid levels (Romero, 2004; Sapolsky et al., 2000). As previously mentioned, the vertebrate stress response is under genetic control from the *CRF* and *POMC* genes (Drouin, 2016), and the transcription and translation of *POMC* is the precursor of numerous peptides, including ACTH, α-MSH, β-MSH, γ-MSH, and δ-MSH (Harno et al., 2018). Subsequently, these melanocortin peptides are agonists to the melanocortin receptors (MCR 1–5), which, in addition to stress physiology influence diverse phenotypic traits (e.g., pigmentation, appetite, energy expenditure, immune function; Cone, 2006; Ducrest et al., 2008; Gantz & Fong, 2003). In total, we found support for sex- and treatment-dependent gene expression patterns following our *a priori* hypotheses in four cases (*POMC II* in brain and kidney, *MC3R* in kidney and *MC4R II* in brain), i.e., the predator treatment influenced gene expression profiles differently in males and females. In all these cases, predator exposure resulted in greater expression in males compared to females, while the opposite was found among the controls.

First, in line with earlier studies on teleost fish, we found three different gene copies of *POMC* (Kang & Kim, 2015; Leder & Silverstein, 2006; Takahashi et al., 2005); one copy (*POMC II*) showed clear sex-dependent treatment effects on expression in both the brain and kidney. In general, the *POMC* gene is viewed to have its major expression pattern in the pituitary and hypothalamus (Drouin, 2016). Our results for *POMC II* brain expression may thus reflect sex-specific stress responses to predator exposure, potentially influencing the inducible morphological defence. However, due to its multifaceted effects on phenotypic processes it can be widely expressed across different tissues (Cardoso et al., 2011; Harno et al., 2018). A great example is the pivotal role of UV-radiation induced *POMC* expression in skin melanocytes, driving the melanin synthesis (Chakraborty et al., 1996; Costin & Hearing, 2007). Yet, little is currently known about *POMC* expression in the kidney (Cardoso et al., 2011). Because biosynthesis of glucocorticoids are performed by interrenal cells in the head kidney of teleost fishes (Mommsen et al., 1999), higher levels of melanocortin peptides may increase stress responses, even from *POMC* expression in the kidney. While effects of *POMC* expression in the kidney need further study, the observed upregulation of *POMC II* expression among predator-exposed males, corresponding to their induced body morphology, suggests a proximate link between physiology and morphology in crucian carp.

Second, two of the melanocortin receptor genes had the same sex- and treatment-dependent expression pattern as observed in the *POMC II* gene. Both MC3R (kidney) and MC4R (brain) are activated by multiple peptides, including α-MSH and ACTH (Cone, 2006). Furthermore, both MC3R and MC4R are involved in energy homeostasis and known to influence appetite and growth (Coll & Loraine Tung, 2009; Ducrest et al., 2008; Gantz & Fong, 2003). Intriguingly, earlier studies on crucian carp have shown that predator exposure reduces activity levels (Holopainen et al., 1997a; Vinterstare et al., 2020a), respiration and heart rate but increases overall growth rate (Holopainen et al., 1997a) where a striking increase in muscle mass can be observed (Domenici et al., 2008). We suggest that this change in energy homeostasis may be proximately linked to our findings of predator-induced expression of *MC3R* and *MC4R II* and could influence body morphology through effects such as altered feeding, activity, and muscle growth. Furthermore, α-MSH can modulate the stress response by binding to MC4R, resulting in reduced levels of circulating glucocorticoid concentrations, a process assumed to generate a greater resistance to stressors (Chaki et al., 2003; Racca et al., 2005). Since the constant presence of predator cues may cause a chronically stressful situation for prey, with glucocorticoid concentrations above baseline levels (Balm & Pottinger, 1995; Boonstra, 2013; Clinchy et al., 2013; Hammerschlag et al., 2017), an improved stress resistance from higher *MC4R* expression may be advantageous by reducing the deleterious effects of chronic stress (Boonstra,

2013; Romero, 2004). This may be particularly important for males in teleost fish, since earlier studies have shown that the stress response of males is significantly stronger than the stress response of females (Rambo et al., 2017; Vinterstare et al., 2021).

Due to the complexity of the stress response and the many diverse peptides cleaved from the precursor POMC (Harno et al., 2018), altered stress levels may result in multifaceted effects on the phenotype; experimental manipulation of cortisol concentrations in fish influence growth rate (Bernier et al., 2004; De Boeck et al., 2001; Midwood et al., 2014), reproductive function (Carragher et al., 1989; Crossin et al., 2016), behavior (Barreto et al., 2014), and appetite (Bernier & Peter, 2001; Bernier et al., 2004). Of importance here is the negative feedback loop of the stress axis, where excessive levels of glucocorticoids inhibit transcription of *POMC* (Birnberg et al., 1983; Drouin et al., 1989; Sapolsky et al., 2000). For example, experimental addition of cortisol is known to repress the stress axis in goldfish (*Carassius auratus*), a species closely related to crucian carp (Bernier et al., 1999; Fryer et al., 1984). As mentioned earlier, POMC-derived peptides are not only involved in the stress axis, they are also central in the melanocortin system and regulate melanin-based pigmentation (Leclercq et al., 2010; Sugimoto, 2002). This pleiotropy of POMC creates a proximate bridge between numerous phenotypic aspects, from external characters such as body colouration to differences in behavioral syndromes (Ducrest et al., 2008; Höglund et al., 2000). For example, a recent study showed that predator exposure caused a dramatic darkening of body colouration in crucian carp (Vinterstare et al., 2020b). In teleost species, physiological stress is known to enhance melanogenesis, resulting in skin darkening from enhanced levels of melanocortins (α-MSH and ACTH) directly derived from stress-induced upregulation of *POMC* (Höglund et al., 2000; Leclercq et al., 2010). In line with this, anti-predator traits (body coloration and body depth) in crucian carp are negatively influenced by intraperitoneal implants containing cortisol (Vinterstare et al., 2020b)—we propose this is proximately caused by cortisol-repression of *POMC* from the negative feedback system of the vertebrate stress axis (Drouin et al., 1989; Sapolsky et al., 2000). These earlier results, along with the expression of sexual dimorphism in morphological defences and stress gene profiles shown here, clearly indicate a sex-specific major role of physiological stress in regulating the inducible morphological defence. The higher expression of *POMC* among predator-exposed males suggests a higher level of predator-induced stress in male fish, and, thus, that such increased stress may drive the sexual dimorphism in the anti-predator phenotype. Meanwhile, the upregulation of the *MC4R* gene in predator-exposed males may reflect a mechanism to cope with this greater stress activity (Chaki et al., 2003; Racca et al., 2005). Patterns of sexual dimorphism in stress physiology have recently been shown to have evolved in other teleosts, such as Bahamas mosquitofish (Vinterstare et al., 2021). In regard to sexual dimorphism in stress physiology, some researchers have even argued that the "fight-or-flight" response is from an evolutionary perspective particularly important in males, since females of many species are significantly less aggressive and that selection would rarely favor the option of flight in a mother having dependent offspring (Taylor et al., 2000). Instead, it has been argued that female responses to stress are founded on processes related to attachment and caregiving processes that ultimately would downregulate the HPA/HPI axis. Hence, instead of a fight-or-flight response, females of many species may respond to stress by a "tend-and-befriend" response mediated by oxytocin and regulated by sex hormones and endocrine mechanisms (Sapolsky et al., 2000; Taylor et al., 2000).

In sum, our results show that predation risk induces strong sex-dependent variation in morphology in an otherwise monomorphic vertebrate, indicating the importance of non-lethal predation risk as a potent driver for phenotypic variation in nature. Furthermore, by incorporating classic life-history theory we provide novel insight into the potential mechanisms underlying sex-specific strategies in coping styles against predation, i.e., sex-specific investments into different evolutionary strategies. Lastly, our data suggest a correlative link between the sex-specific differences in inducible morphological defence expression and expression patterns of the genetic pathway of physiological stress.

Supplementary material

Supplementary material is available online at *Evolution* (https://academic.oup.com/evolut/qpac030)

Data availability

Vinterstare, Jerker (2022). Sex matters: predator presence induces sexual dimorphism in a monomorphic prey, from stress genes to morphological defences. Dryad, Dataset, https://doi.org/10.5061/dryad.x3ffbg7p5.

Ethical statement

Ethical permit for care and use of experimental animals were followed and provided by the Malmö/Lund Ethical Committee (M36-14).

Author contributions

J.V. developed the original idea and designed the study. J.V. performed the laboratory experiment and collected all field data. J.V. extracted morphological measurements from digital photographs and R.B.L. performed the morphometric and statistical analyses and produced the figures and tables together with J.V. P.C. did the bioinformatics. J.V. led the writing and revisions and K.H., R.B.L., P.A.N., P.C., B.H., and C.B. contributed substantially to the final manuscript. This project was funded by the Swedish Research Council to C.B.

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Supplementary materials for **Sex matters: predator presence induces sexual dimorphism in a monomorphic prey, from stress genes to morphological defence expression**

Table S1. Summary of the percent of total variance in body shape explained by each axis in the Relative Warps Analysis conducted on 425 crucian carp. Note that the first axis captured the bulk of shape variation.

Relative Warp Axis	Percent Variance Explained
RW1	61.12
RW2	14.08
RW3	5.56
RW4	3.34
RW5	2.78
RW6	2.21
RW7	2.08
RW8	1.87
RW9	1.51
RW10	1.19
RW11	0.91
RW12	0.83
RW13	0.74
RW14	0.54
RW15	0.39
RW16	0.35
RW17	0.28
RW18	0.24

Supplementary morphological analyses

Conducting Generalized Procrustes Analysis (GPA) and Relative Warps Analysis (RWA) separately for the 366 fish collected in the wild from two lakes (Bergundasjön and Häckebergasjön), we retained the first 10 RWs, explaining 91.46% of shape variation, for analysis. Using these 10 RWs as dependent variables in a MANCOVA following that described in the main text, we found very similar patterns as presented in the main text, with sexual dimorphism in body shape strongly apparent (Table S2). Follow-up univariate tests revealed that many RWs showed differences between sexes (RWs 1-4, RW6), but that RW2 had the largest effect size. Moreover, RW2 scores from this analysis were highly correlated with RW1 scores from the analysis presented in the main text ($r = 0.87$, $P < 0.0001$). For these reasons, we visualized variation among sexes and lakes using RW2 (Fig. S2a). These results mirror those presented in the main text, suggesting that our findings were not greatly influenced by whether we examined the field study separately or pooled together with laboratory fish as done in the main text to allow all fish to be visualized on the same axis.

Conducting GPA and RWA separately for the 118 images of fish used in the laboratory experiment (photographs of 59 specimens taken both before and after experimentation), we retained the first 9 RWs, explaining 89.96% of shape variation, for analysis. Using these 9 RWs as dependent variables, we separately conducted mixed-model nested MANCOVAs following that described in the main text for both the "before" and "after" images. Prior to experimentation, we found no clear patterns in the data (Table S3). Even when conducting univariate analyses with each of the 9 RWs, no model term was statistically significant in any case. However, after experimentation we found patterns similar to that reported in the main text (Table S4). Follow-up univariate tests revealed that RW1 largely captured the relevant variation in body shape after the

experiment (strong treatment and interaction effects; only RW3 and RW9 showed additional and weaker treatment effects; only RW6 showed weaker association with the interaction between treatment and sex). For instance, univariate analysis of RW1 scores after experimentation showed clear interaction effects ($P = 0.0159$), while no interaction between sex and predator treatment was observed prior to experimentation ($P = 0.5190$). RW1 scores from this analysis were highly correlated with RW1 scores from the analysis presented in the main text ($r = 0.88$, $P < 0.0001$). For these reasons, we visualized variation among sexes and treatments using RW1 (Fig. S2b). These results largely mirror those presented in the main text, again suggesting that our findings were not affected by whether we examined the laboratory study separately or pooled together with field fish as done in the main text. Further, these results confirmed that differences between treatments did not exist by chance prior to experimentation.

Table S2. Results from MANCOVA examining variation in body shape (10 RWs) of 366 field-collected crucian carp from two lake populations coexisting with predatory fish.

Term	*F*	*df*	*P*
Centroid Size	33.41	10, 351	< 0.0001
Lake	128.16	10, 351	< 0.0001
Sex	16.91	10, 351	< 0.0001
Lake × Sex	1.59	10, 351	0.1080
Lake × Centroid Size	4.63	10, 351	< 0.0001

Table S3. Results from a mixed-model nested MANCOVA examining variation in body shape (9 RWs) of 59 crucian carp prior to laboratory experimentation.

Term	*F*	*df*	*P*
Centroid Size	1.38	8, 209	0.2049
Predator Treatment	0.79	8, 209	0.6092
Sex	1.71	8, 209	0.0976
Predator Treatment × Sex	1.19	8, 209	0.3047

Table S4. Results from a mixed-model nested MANCOVA examining variation in body shape (9 RWs) of 59 crucian carp experimentally raised in either the presence or absence of predator cues.

Term	*F*	*df*	*P*
Centroid Size	2.69	8, 214	0.0078
Predator Treatment	6.14	8, 214	< 0.0001
Sex	1.22	8, 214	0.2887
Predator Treatment × Sex	2.64	8, 214	0.0089

Table S5. Details about the candidate genes of interest.

Seq. Name		Length	Labelled
TRINITY_DN209366_c2_g1	corticotropin-releasing factor receptor 1-like	429	*CRF1*
TRINITY_DN209328_c4_g1	corticotropin-releasing factor receptor 2	479	*CRF2*
TRINITY_DN187869_c0_g1	corticotropin-releasing factor-binding protein	348	*CRF-BP*
TRINITY_DN208882_c1_g2	proopiomelanocortin	222	*POMC I*
TRINITY_DN192709_c1_g2	pro-opiomelanocortin B-like	238	*POMC II*
TRINITY_DN192709_c1_g3	pro-opiomelanocortin B-like	267	*POMC III*
TRINITY_DN196455_c3_g1	melanocortin-2 receptor accessory protein 2A	214	*MRAP2A*
TRINITY_DN185506_c4_g2	melanocortin-2 receptor accessory protein 2B	202	*MRAP2B*
TRINITY_DN208669_c2_g1	melanocortin receptor 3-like	325	*MC3R*
TRINITY_DN201926_c4_g2	melanocortin 4 receptor	326	*MC4R I*
TRINITY_DN192183_c3_g1	melanocortin receptor 4-like	205	*MC4R II*
TRINITY_DN201926_c4_g1	melanocortin receptor 5-like	334	*MC5R I*
TRINITY_DN201926_c4_g5	melanocortin receptor 5-like	353	*MC5R II*

Table S6. Distributions fitted to the generalized linear models described in the main text, selected using AIC_c.

Gene	Brain	Kidney
CRF1	Normal	Normal
CRF2	Normal	Normal
CRF-BP	Poisson	Poisson
POMC I	Poisson	Poisson
POMC II	Poisson	Poisson
POMC III	Normal	-
MRAP2A	Normal	Normal
MRAP2B	Poisson	Poisson
MC3R	Poisson	Poisson
MC4R I	Normal	-
MC4R II	Normal	Poisson
MC5R I	Normal	-
MC5R II	Poisson	-

Figure S1. Illustration of the eleven fixed landmarks used to examine the effect of predator exposure on male and female crucian carp morphology.

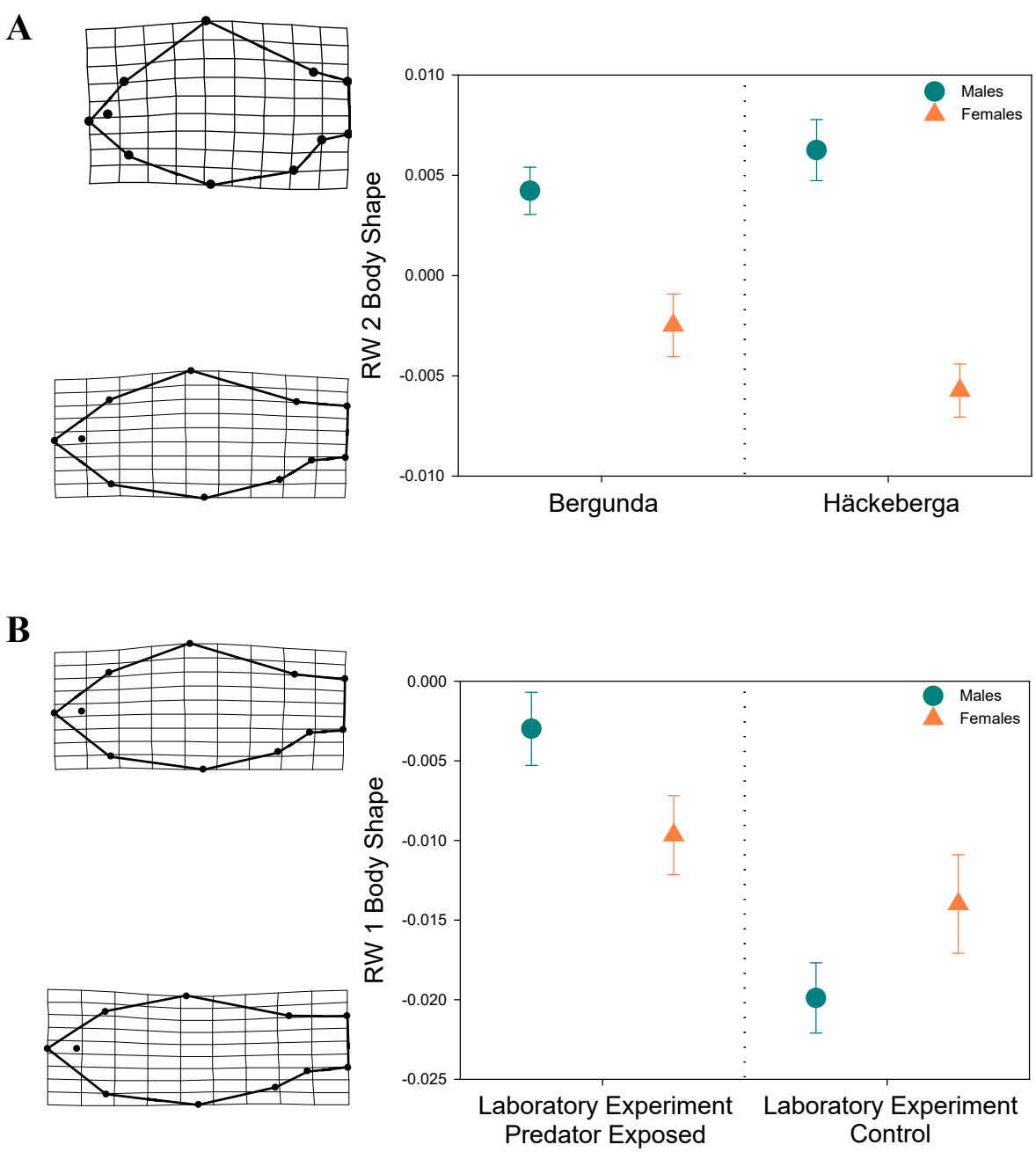

Figure S2. Variation in body shape among males and females (A) in two lake populations coexisting with predatory fishes and (B) when exposed/unexposed to predator cues in a laboratory experiment. Thin-plate spline transformation grids illustrate the observed ranges of body shapes within each dataset along the respective Relative Warp, with lines drawn to aid interpretation of the body outline of the fish. Least-squares means ± SE depicted.

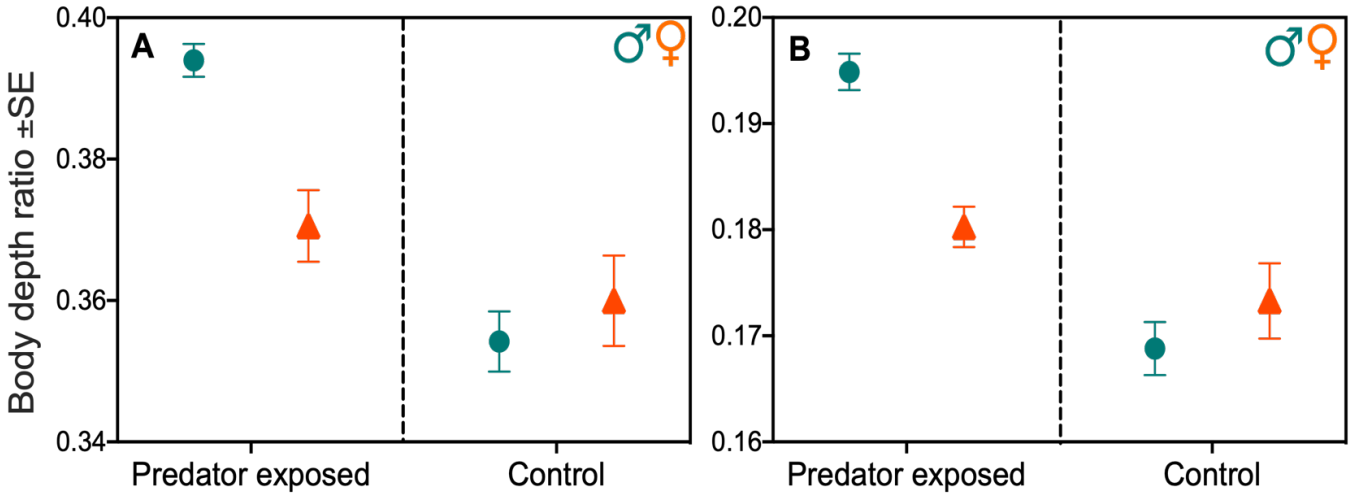

Figure S3. In addition to the landmark-based morphometrics, we here also illustrate the (A) total body depth and (B) the lateral line body depth, i.e., the full/half distance for the dorsoventral axis for experimental subjects from our plasticity experiment. Furthermore, we analysed these data set using GLM:s, with treatment and sex as fixed factors, body depth and lateral line body depth as dependent variables and the standard length of fish as a covariate. Tank identity was used as a random factor, nested within treatment. Result for (A) total body depth: sex x treatment interaction term: $F_{1.42} = 4.595$, $p = 0.038$; result for (B) lateral line body depth: sex x treatment interaction term: $F_{1.42} = 8.332$, $p = 0.006$.

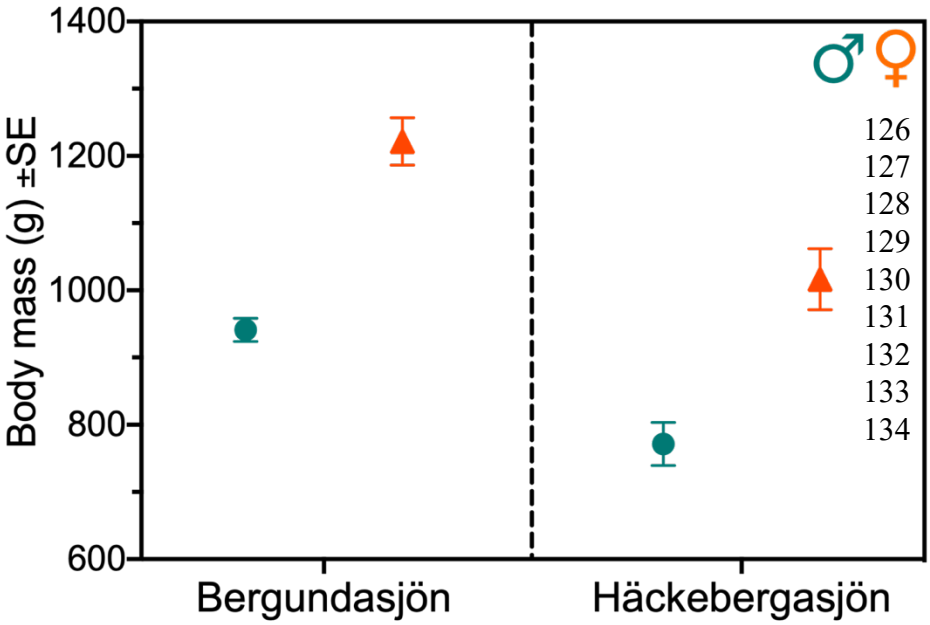

Figure S4. Mean body mass (weighed to nearest 0.1 g) for wild-caught male and female crucian carp from two lakes with constantly high predation risk. Furthermore, we performed univariate analysis of variance for each data set obtained from the two lakes with the factor sex as the fixed factor and body mass (in gram) as the dependent variable. Result for Bergundasjön: $F_{1.195} = 59.569$, $p < 0.001$; result for Häckebergasjön: $F_{1.165} = 15.266$, $p < 0.001$.

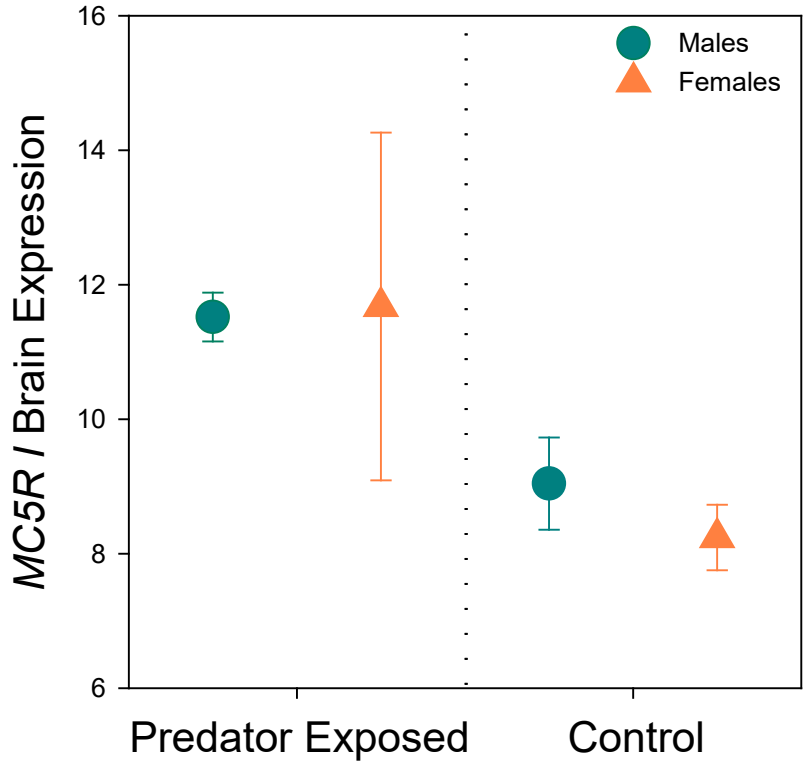

Figure S5. Normalized gene expression of *MC5 I* in the brain of crucian carp in the laboratory experiment. Both sexes showed greater expression in the predator treatment. Means ± SE depicted.

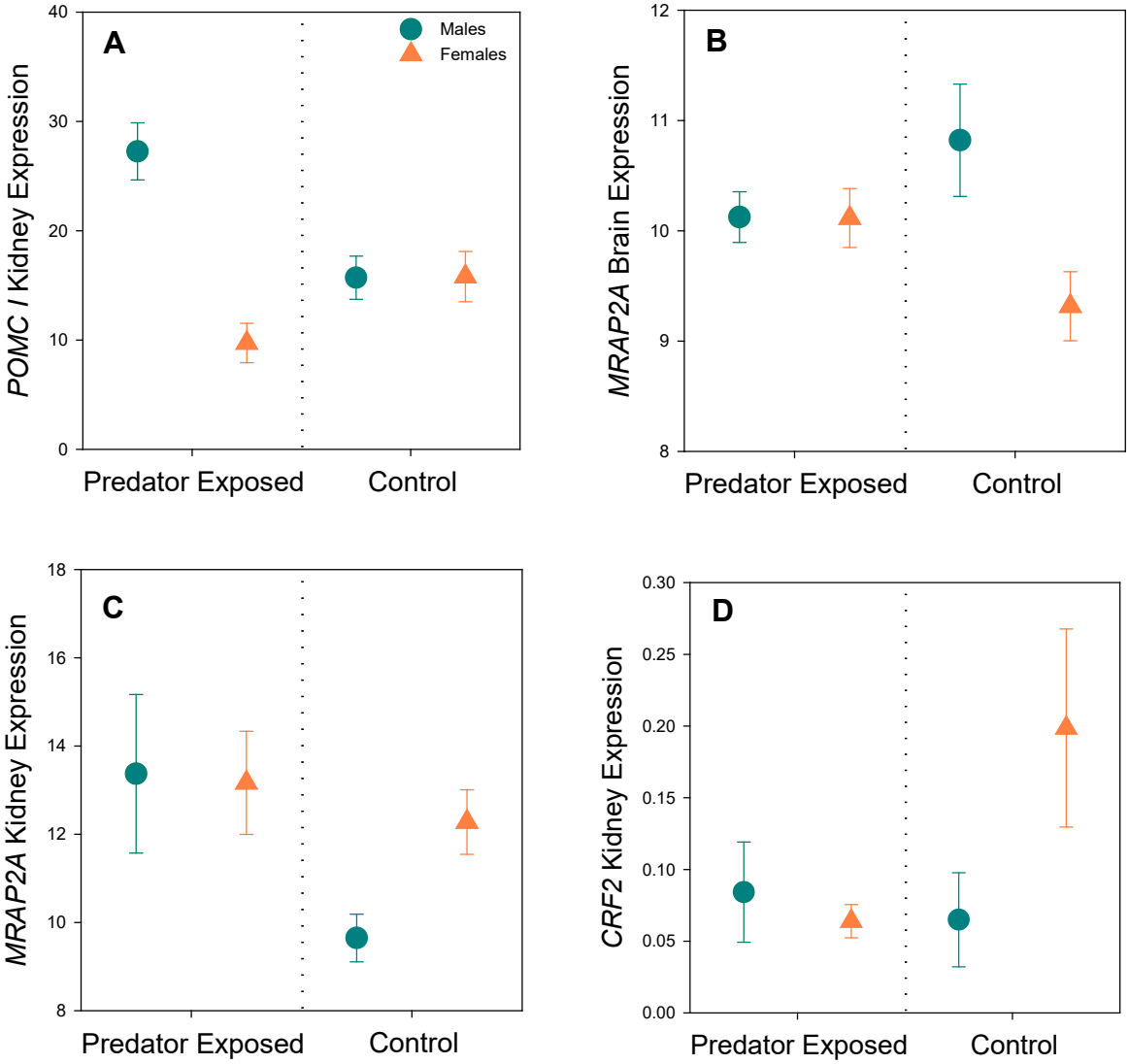

Figure S6. Normalized gene expression of crucian carp across sexes and predator treatments for the four cases of suggestive trends that did not remain significant after FDR adjustment (see table 3 in the main text). Means ± SE depicted.