Meta-analysis shows environmental contaminants elevate cortisol levels in teleost fish – effect sizes depend on contaminant class and duration of experimental exposure

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Highlights:

- Does exposure to environmental contaminants (ECs) affect fish stress responses?
- Studying fish responses to ECs is important for conservation.
- Study lies at intersection of anthroposphere and biosphere.
- Meta-analysis shows generally elevated fish cortisol, dependent on class of EC.
- EC exposure duration affects fish cortisol to particular ECs.

Abstract

Glucocorticoid hormones (GCs) help vertebrates maintain homeostasis during and following challenging events. Short-term elevations in GC levels are necessary for survival, whereas longer-term changes can lead to reduced reproductive output and immunosuppression. Persistent environmental contaminants (ECs) are widespread globally. Experimental exposure of individuals to ECs is associated with varying GC responses, within, and across, species and contaminants. Individuals exposed to ECs over long durations are expected to have prolonged GC elevations, which likely affect their health. We conducted a meta-analysis to test for a relationship between fish GC levels and experimental exposure to ECs, and to explore potential moderators, including duration of exposure, that could help explain the variation in effect sizes within and between studies. We report almost exclusively on cortisol responses of teleost fish to ECs. Although there was much variation in effect sizes, captive-bred fish exposed to ECs had baseline GC levels 1.5X higher than unexposed fish, and fish exposed to pharmaceuticals (estradiols and stimulants being mainly considered) had baseline GC levels approximately 2.5X higher than unexposed fish. We found that captive-bred and wild-caught fish did not differ in GC levels after exposure to the same classes of ECs - studies on captive bred fish may thus enable inferences about GC responses to ECs for wild species. Furthermore, effect sizes did not differ between baseline and challenge-induced GC measures. In different analyses, duration of exposure was negatively correlated to effect size, suggesting that the GC response may acclimate after chronic exposure to some ECs which could potentially alter the GC response of EC-exposed fish to novel stressors. Future studies should explore the effect of multiple stressors on the fish GC response and perform tests on a broader array of contaminant types and vertebrate classes.

Key Words: Stress hormones, HPI axis, exposure duration, pollutants, response ratio, stressor

1. Introduction

Glucocorticoids (GCs) are hormones synthesized in the adrenal glands of vertebrates that are released into circulation when the animal experiences a challenging stimulus (e.g., predator encounter, environmental perturbation) (Romero, 2004; Sapolsky et al., 2000). Circulating GCs help the animal maintain homeostasis during, and following, a stressful event by mediating certain cardiovascular, digestive, behavioural, and metabolic functions through permissive, suppressive, stimulatory and/or preparative actions (Sapolsky et al., 2000). Short-term changes to GC levels are necessary for survival, whereas long-term changes can have negative effects on the animal's health and/or reproduction.

Long-term elevation of GCs can reduce reproductive effort. For example, Schoenle et al., (2017) showed that elevated levels of GCs were associated with lower incubation effort and smaller clutch sizes in red-winged blackbirds (*Agelaius phoeniceus*). Additionally, GCs have suppressive effects on the immune system; secreted GCs work to prevent the immune and inflammatory responses from overshooting (Sapolsky et al., 2000). While short-term increases of GCs are important for mediating the immune response, long-term increases of GCs can lead to increased disease susceptibility and/or intensity of infection (Belden & Kiesecker 2005; Fonner et al., 2017). Alternatively, if secretion of GCs is impaired over the long-term, the ability of a vertebrate to respond appropriately to a challenge may be inhibited (Hontela et al., 1995). Thus, long-term exposure to stressors resulting in prolonged elevation or depression in GC levels can have detrimental effects on vertebrate health and/or reproduction.

Environmental contaminants (ECs) are one category of potential stressors on vertebrates. There are many forms of ECs (e.g., pharmaceuticals, pesticides, heavy metals, plastics) and their widespread occurrence in the environment is highly associated with anthropogenic activities

(Browne et al., 2011; de Souza et al., 2020; Driscoll et al., 2013; Ebel et al., 2017; Hayes and Hansen, 2017; Stoiber et al., 2020). ECs can be persistent and are relatively ubiquitous in the environment (reviewed in Ebel et al., 2017 (pharmaceuticals); Li et al., 2020 (per- and polyfluoroalkyl substances); de Souza et al., 2020 (pesticides)); therefore, there is widespread potential for organisms to be exposed to ECs over prolonged periods of time. Prolonged exposure of non-target vertebrate organisms to ECs could result in long-term changes to circulating GCs. The extent to which experimental exposure to ECs affect GCs generally for vertebrates, or even specifically for any vertebrate class, has not been assessed comprehensively across studies. This lack of synthesis is surprizing given ECs are suspected of being environmental stressors and GC responses are thought to reflect stress responses (MacDougall-Shackleton et al., 2019). Herein, we used meta-analytical approaches and their associated rigorous literature searches to provide a synthesis of the effects of ECs on GC responses (more specifically cortisol) of fish.

The effects of EC exposure on GC levels have been measured across multiple vertebrate groups both in laboratory and field settings with different patterns reported. Many studies have found that GC levels increase with exposure to ECs. For example, Adams et al., (2009), found that postfledging white ibises (*Eudocimus albus*) exposed to dietary methylmercury had higher fecal GC levels compared to control ibises. Similar patterns have been documented in Nile tilapia (*Oreochromis niloticus*) exposed to heavy metals (Firat and Kirgan, 2010). Increases in amphibian or fish GC levels occur with other classes of contaminants such as polybrominated diphenyl ethers (Freitas et al., 2017), pesticides (McMahon et al., 2011), and pharmaceuticals (Sebire et al., 2015). In contrast, other organisms exposed to ECs secrete lower levels of GCs suggesting an inability to mount an appropriate stress response. Francheschini et al., (2017), showed that juvenile common loons (*Gavia immer*) exposed to methylmercury exhibited a decreased ability to mount a stress

response compared to unexposed juveniles. Impairment of the GC response has been documented in American kestrels (*Falco sparverius*) exposed to polychlorinated biphenyls (Love et al., 2003), wild house sparrows (*Passer domesticus*) exposed to crude oil (Lattin et al., 2014), and zebra finches (*Taeniopygia guttata*) exposed to mercury (Moore et al., 2014). Still, other studies have reported no effect of EC exposure on the vertebrate GC response (e.g., Maddux et al., 2015). Such variation in GC responses for vertebrates exposed to ECs suggests that moderating factors, such as species or taxa, life stage, contaminant type and/or duration of exposure, might be important in determining the direction and strength of the responses.

This study focuses on whether there are overall patterns in GC responses to different ECs for one group of vertebrates: fish. We conducted a meta-analysis on the general relationship between fish GC levels and experimental EC exposure and investigated potential moderator variables that could help explain the variation in responses observed within and between studies. Moderators included class of ECs, whether fish were wild caught or sampled from breeding stock, whether baseline versus challenge-induced GC levels were assayed, and the concentration and duration of exposure to ECs. We also explicitly considered shared evolutionary history of test species. We chose to focus on fish species only because our literature search returned too few effect sizes for other wildlife species which would have to be further subdivided by contaminant class, captive bred versus wild caught, and baseline versus challenge-induced measures.

We compared wild-caught and captive-bred test subjects to determine if studies involving the latter might enable inferences about GC responses to ECs for wild-caught species, temporarily housed in the lab. Vertebrates tend to secrete lower levels of GCs upon repeated exposure to the same stressor (e.g., handling), resulting in acclimation to the stressor (Dobrakovova et al., 1993). Therefore, captive-bred vertebrates that are repeatedly exposed to stressors such as handling, or

93 confined spaces, may be expected to secrete GCs at different levels compared to wild-caught
94 vertebrates of the same species when first exposed to the same challenges. Notably, Kunzl and
95 Sachser (1999) found that domesticated guinea pigs (*Cavia aperea f. porcellus*) had significantly
96 reduced secretion of GCs in response to a stressor compared to their wild ancestors, the cavy
97 (*Cavia aperea*). Thus, domestication or captivity may significantly alter the stress response of
98 vertebrate organisms.

99 We compared effect sizes based on baseline versus challenged-induced GC measures,
100 parsing data by contaminant class. In so doing, we determined if quantitatively different responses
101 for these different arms of the hypothalamic-pituitary-adrenal/interrenal (HPA/HPI) axis response
102 were occurring. Qualitatively, these responses engender different physiological actions. At
103 baseline concentrations, GCs exert permissive actions and at challenge-induced levels, GCs exert
104 stimulatory, suppressive, and/or preparative actions (Romero, 2004; Sapolsky et al., 2000). The
105 two types of GC responses (baseline and challenge induced) provide different information on the
106 stress response of vertebrates and should be considered separately in analyses but can be compared
107 for magnitude of EC-related effects. In teleost fish (the main subjects of the current study), cortisol
108 is the main glucocorticoid and can bind to both mineralocorticoid receptors (MRs), responsible for
109 regulating hydromineral balance, and glucocorticoid receptors (GRs), responsible for actions such
110 as energy mobilization (Wendelaar Bonga, 1997).

111 Using meta-analysis and the PRISMA methodology (Liberati et al., 2009), we summarize
112 the current knowledge of the effects of ECs on the fish GC response and further help identify
113 knowledge gaps which need to be addressed in future research to improve the understanding of
114 how ECs influence 'stress responses' of fish and other wildlife.

115 **2. Methods**

116 *2.1 Literature searches*

117 Our intention was to conduct literature searches to provide data to determine the
118 relationship between ECs and GC responses in wildlife, including fish. The first of two literature
119 searches was performed on November 4th, 2019 using the Web of Science: Core Collection
120 database and the following key terms in a general topic search (title/abstract/key words):
121 (toxicology OR ecotoxicology OR contaminants) AND (glucocorticoid* OR corticosteroids OR
122 corticosterone OR cortisol OR cort*). The initial search provided 1,128 records. The second
123 literature search was performed on December 2nd, 2019 using the Scopus database and the same
124 key terms as provided above. Upon initial examination, many of the studies included human trials
125 and clinical studies; therefore, we chose to add a filter that excluded the subject area of medicine.
126 We excluded human studies from further consideration because we were focusing on the effects
127 of ECs on GC responses in wildlife. Our final search for the Scopus database was as follows:
128 TITLE-ABS-KEY ((toxicology OR ecotoxicology OR contaminants) AND (glucocorticoid* OR
129 corticosteroids OR corticosterone OR cortisol OR cort*)) AND (EXCLUDE (SUBJAREA,
130 "MEDI")). This search provided 1,598 records. We did not restrict the publication date or the
131 journal of publication for either search. Once duplicates were removed (n=540) we had a pool of
132 2,184 records to screen.

133 *2.2 Screening studies and data extraction*

134 Initially, we screened studies by their abstracts and titles. We removed any study that did
135 not have a clear EC focus, or did not mention GCs or hormone testing, or was not reporting on GC
136 responses of vertebrate species (e.g., invertebrate exposures, cell line exposures). We also
137 excluded studies that did not directly measure GC titre (e.g., measured GC receptor expression),
138 or which were not experimental (e.g., review paper). A total of 1,944 records were excluded during

initial screening. Eligibility assessments were conducted for the remaining 240 studies by reviewing the full text. For a study to be retained, it had to be an experimental study conducted in a controlled setting (i.e., laboratory), and the substance evaluated had to be a known or potential EC. Further, the study had to provide the mean and variation for the relationship between EC exposure and GC levels, and an unexposed group (control) had to be present. We omitted any study using lab-bred mice and rats due to the presence of significant inbreeding after generations of laboratory housing (Lutz et al., 2012; Silver, 2001) which would not be widely applicable to wild populations. Two studies were excluded due to the exposures being conducted on eggs - the HPI axis of the study organism was not developed (Barry et al., 1995; Jentoft et al., 2002, Tsalafouta et al., 2014). Early on, we determined that only the fish group had sufficient replication to address our study objectives, so we chose to only include studies using fish as test subjects. The final eligibility screening resulted in 44 studies being retained for the meta-analysis (Figure 1). References for included studies are provided in Supplementary Information. With one exception (Miandare et al., 2005), the test subjects were teleost fish species. And with the exception of one other study (Gay et al., 2013), the GC measured was cortisol. As such, our study is largely restricted to cortisol (also referred to as GC) responses of teleost fish to experimental exposure to ECs.

From each of the 44 studies, we recorded the year of study, sex and life-history stage of the test species, whether the organisms were wild-caught or captive-bred, contaminant name (e.g., mercury, atrazine), contaminant class (e.g., metal, pesticide, pharmaceutical), exposure concentration(s), duration of the exposure (or time of exposure to EC when GC measure was taken), where GC concentrations were collected from (e.g., plasma, water, whole-body), type of GC measure (baseline or stress-induced), and the mean and variation of GCs extracted from each

exposure group. Data presented in figures were extracted using the GetData Graphic Digitizer (Fedorov, 2013). We extracted data for single-compound exposures and omitted any data on exposures to contaminant mixtures. We did not extract data for GC measures sampled during or after EC exposure recovery periods, as recovery time can affect GC concentrations (Ruiz-Jarabo et al., 2019; Ruiz-Jarabo et al., 2020), and we were interested in understanding the direct effects of EC exposure on teleost fish cortisol responses. We used solvent control data in cases where both a water and solvent control were used because using the solvent control reduces the likelihood of false negative treatment effects (Green and Wheeler, 2013).

2.3 Calculating effect sizes

Effect sizes were calculated as the natural log response ratio (LRR): $\ln[RR] = \ln[X_E/X_c]$ where X_E is the experimental (exposed) group mean, and X_c is the control (unexposed) group mean, with variance $v = (SD_E)^2/N_E X_E^2 + (SD_C)^2/N_C X_C^2$, where SD_E is the standard deviation of the experimental group, SD_c is the standard deviation of the control, N_E is the experimental group sample size, and N_C is the control group sample size (Hedges et al., 1999; Lajeunesse, 2011). A positive LRR indicates that EC exposure is associated with increasing vertebrate GC levels, and a negative LRR indicates an association with decreasing GC levels. In the case where there was more than one contaminant present in a study, and/or more than one exposure concentration, an effect size was calculated for each contaminant at each exposure concentration. After effect sizes were calculated, we extracted the largest effect size (negative or positive) from comparisons with repeated measures over time and included these in the final dataset to ensure independence of data points in the overall analysis. Our rationale for choosing the largest effect size was that extreme elevated or depressed responses are important biological responses which are missed when

184 averaging over a range of responses (O'Dwyer et al., 2020). We have provided the final
185 constructed dataset as a searchable and sortable excel file with this article.

186 *2.4 Statistical analyses*

187 All statistical analyses were completed using R version 4.0.2 (R Core Team, 2020). The
188 data were collated by contaminant class and separated by captive-bred vs wild-caught study
189 organisms, and by type of GC measures taken (baseline vs challenge-induced). Thus, fish data
190 were initially split into four categories; captive-bred fish with baseline GC measures; captive-bred
191 fish with challenge-induced GC measures; wild-caught fish with baseline GC measures; and wild-
192 caught fish with challenge-induced GC measures (Table 1).

193 We conducted generalized linear mixed models (GLMMs) using the '*MCMCglmm'*
194 package (Hadfield, 2010) to examine the relationship between EC exposure and fish GC response.
195 We included species identity and study identity as random effects. To account for phylogenetic
196 relatedness in our models, we included phylogenetic trees for the fish species included in the
197 different analyses (Figure S1, S2, S3). We generated the phylogenetic trees using PhyloT
198 (phylot.biobyte.de/). PhyloT generates trees using data from the National Centre for
199 Biotechnology Information database and the Genome Taxonomy Database. We resolved
200 polytomies in the trees using the following cited sources (Betancur et al., 2013; Crete-Lafreniere
201 et al., 2012; Rabosky et al., 2018; Tang et al., 2011).

202 We fit GLMMs using a Markov Chain Monte Carlo (MCMC) algorithm with a weakly
203 informative prior (V (variance) = 1, nu (degree of belief parameter) = 0.002) for the (co)variances
204 and the MCMCglmm default prior for the fixed effects. The MCMC method samples from a
205 posterior distribution and provides the mean and credible interval (CI) of that distribution. When
206 the 95% CI does not cross 0, the posterior distributions reported are considered statistically

supported. The models were run using 13000*20 iterations, a thinning interval of 10*15, and a burnin of 3000*10, with one exception; the model examining differences between captive-bred and wild-caught fish exposed to ECs required an alternative set up of 13000*40 iterations, a thinning interval of 10*15, and a burnin of 3000*20. Models were run ensuring that sampling effort exceeded 1000 in all instances. We assessed the level of non-independence between successive samples in the chain taken from the posterior distribution using the '*autocorr'* function in the '*MCMCglmm'* package (Hadfield, 2010). We assessed model convergence by visually inspecting trace plots of the parameters. Heterogeneity was measured using I^2. Consideration of high heterogeneity is made alongside of consideration of moderator variables that might explain some of that heterogeneity. Moderate to high heterogeneity is expected in ecological meta-analyses; Senior et al., (2016), reported an average I^2 heterogeneity of 91.69% from 700 ecological and evolution meta-analyses. To assess publication bias, we visually inspected a funnel plot of effect sizes and used the Egger's regression test and the trim and fill method in the '*metafor'* package (Viechtbauer, 2010) to test for funnel plot asymmetry.

Our first set of analyses examined the effect of EC exposure on captive-bred fish baseline GC levels for the largest reported GC levels in each study and across all EC classifications. We conducted a basic mixed effects model including duration of exposure and contaminant concentration as fixed effects. The fixed effect of duration of exposure refers to the time during which contaminant exposure was maintained until the GC measure was taken. We included duration of exposure as a predictor of GC responses because prolonged exposure might be expected to dampen any responses. The time that the GC measure was taken (also, duration of exposure) is necessary to include given that GC responses to stressors may not be immediate. For example, in teleost fish GC concentrations have been shown to increase for 1-hr after an acute-

stress challenge (Costas et al., 2011; Skrzynska et al., 2018; Ruiz-Jarabo et al., 2020). Our dataset includes both acute and chronic exposure times and therefore it is important to include duration of exposure to account for variation in GC response over time. Concentration of contaminant was included because responses might be expected to be dose dependent (e.g., no effect of a particular EC might be because of the dose used). Due to large variation in the measurements of duration of exposure and contaminant concentration, the variables were log-transformed, and they were also centred prior to analysis. We then explored additional variables that may have accounted for differences in GC levels. The additional variables examined included life history stage of the fish, type of tissue or media sampled, and year of publication. We compared effect sizes between life history stages first using adults as the reference group to compare with the other groups. Then, we rotated which group was set as the reference group until all possible combinations were assessed. For simplicity, we reported only the results of the first analysis. We used the same method to investigate if there was any difference in effect sizes between tissue/media type sampled, starting with blood as the first reference group. Again, we reported only the results of the first analysis.

We wanted to investigate the influence of EC class on captive-bred fish baseline GC levels, therefore we added EC classification as a fixed effect. We dropped the intercept from the model so that the model output gave each groups' estimated mean and tested whether the group means differed from an effect of zero, rather than a comparison of each EC group to the default reference group "fertilizers" (Schielzeth, 2010). We further excluded continuous predictor variables from the model.

Next, we explored captive-bred versus wild-caught fish GC levels. There were only three EC classifications that were represented across both captive-bred and wild-caught fish studies that measured baseline GC levels (i.e., had one or more effect sizes across both datasets, Table 1).

Therefore, for this analysis only pharmaceutical, pesticide, and polychlorinated biphenyl (PCB) ECs were included. Duration of exposure (centred), contaminant concentration (centred), and captivity status (wild vs captive) were included as fixed effects. This parsing of the data was meant to balance the design somewhat by excluding effect sizes from contaminant classes that had only one type of test subject: wild-caught or captive-bred.

Our final analysis explored baseline GC measures versus challenge-induced GC measures in captive-bred fish exposed to ECs. Only EC classifications that had both baseline and challenge-induced measures were included in the analysis (i.e., metals, polycyclic aromatic hydrocarbons (PAHs), PCBs, pesticides, pharmaceuticals, and phenols, Table 1). Duration of exposure (centred), contaminant concentration (centred), and type of GC measure (baseline vs challenge-induced) were included as fixed effects. Here again data was parsed to balance the design, excluding effect sizes from contaminant classes that had measures for only one of either baseline or challenge-induced.

3. Results

3.1 Data Summary

We extracted 173 effect sizes, only including the largest reported GC levels from each experiment, from 44 studies. The studies were based on 41 different ECs that were sorted into 11 broad classifications (Table S1). There were 25 species of fish represented in the full dataset (Table S1). As mentioned, fish included were almost exclusively teleostean fish, except for on species, *Acipenser persicus*. Therefore, our results largely pertain to GC responses of teleost fish. Of the 173 effect sizes, 161 were from studies using captive-bred organisms and 12 were based on wild-caught fish, 148 were based on baseline GC levels, and 25 were based on stress- or challenged-induced GC levels (Table S1). As mentioned, all studies measured cortisol levels, except one (Gay et al., 2013) that measured both cortisol and corticosterone, therefore our results largely pertain to

277 the cortisol responses of teleost fish. Duration of exposure to ECs ranged from 1 h to 130 d,
278 averaging 389.88 ± 531.93 h (mean ± SD). Acute exposures for teleost fish are considered
279 exposures lasting from 2 to 192 h, with chronic exposures being those that last over 2 w (Arjona
280 et al., 2007; Laiz-Carrion et al., 2005). Therefore, 89 effect sizes belonged to the acute exposure
281 category with the remaining 84 effect sizes belonging to the chronic exposure category. The
282 contaminant concentrations ranged from 2.00 X 10^{-5} ppm to 500 ppm, averaging 9.73 ± 55.55
283 ppm. Visual inspection of the funnel plot suggested that no publication bias was present
284 (symmetrical shape) and the Egger's Regression test was non-significant, also suggesting no
285 publication bias was present in our dataset (Egger's regression z = -0.3459, p = 0.7294, Figure S4).
286 The trim and fill method suggested that very few studies were missing from the right side of the
287 plot, however the test of the null hypothesis that the number of missing studies on the right side is
288 zero was not significant (3 studies SE = 2.8284, p = 0.0625). Therefore, the results suggest no
289 publication bias is present in our dataset.

290 *3.2 Baseline GC levels of captive-bred fish exposed to ECs*

291 The dataset based on captive-bred fish with baseline GC measures included 33 ECs across
292 11 classifications, for 19 fish species. Duration of exposure to the contaminants ranged from 6 h
293 to 130 d, averaging 387.55 ± 580.17 h. The contaminant concentrations ranged from 2.00 x 10^{-5}
294 ppm to 500 ppm, averaging 11.89 ± 62.26 ppm.

295 Fish GC levels increase after experimental exposure to ECs (posterior mean = 0.430, 95
296 %, credible interval (CI) = 0.052 to 0.811, Table 2, Figure 2). There was no evidence that duration
297 of exposure or contaminant concentration influenced GC baseline levels in exposed fish (Table 2),
298 however there was a general trend of decreasing effect size with increasing duration of exposure
299 (Figure S5). Heterogeneity arising from phylogeny and species identity was low (I^2 = 4.72 % and

300 3.70 % respectively, Table S2), and heterogeneity among studies was moderate (I^2 = 55.20 %,
301 Table S2).

302 The second mixed model showed that fish exposed to compounds belonging to the
303 pharmaceutical EC classification (Table S1) had higher GC levels than unexposed fish (posterior
304 mean = 0.902, 95 % CI = 0.057 to 1.650, Table 3). We found no evidence that the other 10 EC
305 classifications consistently influenced GC levels in exposed fish. There was no difference in
306 captive-bred fish baseline GC levels between life-history stages or media/tissue sample types
307 based on every possible group comparison (Table S3, S4). There was no influence of year of
308 publication on captive-bred fish baseline GC levels (Table S5).

309 *3.2 Baseline GC levels of captive-bred versus wild-caught fish exposed to ECs*

310 The dataset included 18 ECs across three contaminant classes for 14 species of fish.
311 Duration of exposure to the contaminants ranged from 24 h to 130 d, averaging 545.74 ± 620.27
312 h. The contaminant concentrations ranged from 2.00 x 10^{-5} ppm to 500 ppm, averaging 18.35 ±
313 86.39 ppm.

314 There was no evidence that baseline GC levels of captive-bred and wild-caught fish
315 differed (Table 4, Figure S6). Duration of exposure (centred) influenced fish GC levels after
316 exposure to ECs (posterior mean = -0.271, 95 % CI = -0.481 to -0.047, Table 4, Figure 3). There
317 was a negative relationship between effect size and duration of exposure, i.e., GC levels went down
318 with increasing exposure duration. Heterogeneity arising from phylogeny and species identity was
319 low (I^2 = 15.58 % and 12.43 % respectively, Table S6) and heterogeneity among studies was
320 moderate (I^2 = 44.44 %, Table S6). Total heterogeneity was high (I^2 = 97.74 %, Table S6).

321 *3.3 Baseline vs challenge-induced GC levels of captive-bred fish exposed to ECs*

The dataset included 31 ECs across six classifications for 19 species of fish. Duration of exposure to the contaminants ranged from 1hr to 130d, averaging 378.43 ± 509.91hr. The contaminant concentrations ranged from 2.00 x 10^{-5} ppm to 500 ppm, averaging 11.25 ± 60.96 ppm.

There was no evidence that baseline and challenge-induced GC levels differed in captive-bred fish exposed to ECs (Table 5, Figure S7). Duration of exposure (centred) influenced fish GC levels after exposure to ECs (posterior mean = -0.105, 95 % CI = -0.210 to -0.003, Table 5, Figure 3). There was a negative relationship between effect size and duration of exposure: again, GC levels decreased with duration of exposure. Heterogeneity arising from phylogeny and species identity was low (I^2 = 5.38 % and 7.43 % respectively, Table S7) and heterogeneity among studies was high (I^2 = 77.12 %, Table S7). Total heterogeneity was high (I^2 = 99.43 %, Table S7).

4. Discussion

4.1 Effects of ECs on captive bred fish baseline GC responses

Overall, exposure to ECs is associated with an average 1.5X increase in captive-bred fish baseline GC levels compared to unexposed captive-bred fish. Upon closer examination, fish exposed to compounds belonging to the pharmaceutical EC classification in particular showed an average 2.5X increase in baseline GC levels compared to unexposed fish. Pharmaceuticals are prescription or over the counter drugs used to treat or prevent human and animal diseases (Boxall et al., 2012; Ebel et al., 2017). Pharmaceuticals and their metabolites enter the environment through human and animal excrement, discharge from manufacturing industries, the disposal of unused product, wastewater irrigation practices, and via the application of biosolids as fertilizer (Ebel et al., 2017; Taylor and Senac, 2014). The persistence of pharmaceuticals in the environment varies greatly depending on the chemical examined, with some being reported to persist from 8.3 hr to 1200 days (Bu et al., 2016; Lam et al., 2004; Yamamoto et al., 2009). High persistence of

some pharmaceutical compounds shows the potential for long-term exposure leading to long-term increases in GC levels of exposed fish. It is important to note that our dataset only included nine different pharmaceutical compounds and that inferences should be restricted to the subset of compounds studied in this meta-analysis.

The mechanism by which pharmaceuticals could influence the GC response in fish likely varies depending on the compound and level of its persistence. One major compound included in the pharmaceutical classification was 17β-estradiol (E2) (54% of the included effect sizes in this contaminant classification), a steroid hormone often found in oral contraceptive pills, and used for treating symptoms of menopause (Roby, 2019). E2 is a known endocrine disruptor that interferes with sex determination in fish resulting in the development of gonadal abnormalities, and changes in sex ratios and reproduction (Brion et al., 2004; Drastichova et al., 2005; Uguz, 2017). Some previous research would suggest that E2 exposure would be associated with a reduction in fish cortisol concentrations. McQuillan et al., (2003) found that E2 interfered with pregnenolone-supported cortisol synthesis in interrenal tissues of juvenile chinook salmon. However, the same response was not detected in juvenile rainbow trout interrenal tissues after exposure to E2 (McQuillan et al., 2003). Very little literature is available on the potential mechanics related to the effect of E2 on the HPI axis for teleost fish. Future research on the effect of E2 on gene expression, enzyme regulation, and hormone concentrations related to cortisol production and secretion in teleost fish are needed.

While the increase in baseline GCs was more evident for fish exposed to compounds belonging to the pharmaceutical EC classification, general trends toward increasing GC levels were also found for other EC classifications; metals and polycyclic aromatic hydrocarbons (PAHs). However, the remaining EC classifications showed no impact of exposure. This suggests

that other moderator variables might be important predictors of fish GC responses to ECs. Before addressing other moderator variables, we first summarize possible consequences of elevated GC levels in fish.

There are several documented consequences for chronically elevated GC levels in fish. Chronic elevation of cortisol (up to 4 weeks) in fish due to crowding stress has been associated with increased susceptibility to bacterial and fungal diseases, and in turn, increased mortality (Pickering and Pottinger, 1989). Artificial chronic elevation of cortisol has also been linked to significant metabolic changes in fish including chronically elevated circulating glucose levels (Barton et al., 1987), changes to food conversion efficiency (Gregory and Wood, 1999), and lipid accumulation in liver tissue (Jerez-Cepa et al., 2019). Thus, increased fish GC levels due to EC exposure may contribute to reduced overall health and fitness in fish populations. If this is shown to be the case, it is crucial that mitigation measures are introduced to reduce the release of contaminants into the environment.

4.2 Captive-bred versus wild-caught fish

Our results suggest that captive-bred fish studies may enable inferences about GC responses to ECs for wild fish species since we found no difference in GC responses between captive-bred and wild-caught study species. Our results support previous studies showing that GC release rates are similar between wild and captive populations of vertebrates. For example, a recent study conducted using California condors (*Gymnogyps californianus*) found no significant difference in plasma GC levels between wild and captive condors after routine trapping and handling procedures (Glucs et al., 2020). However, other studies have provided contradictory results; Fanson et al., (2012) showed that fecal GC metabolite concentrations were high in captive lynx (*Lynx canadensis*) compared to wild lynx. Another study determined that wild

392 salamanders had significantly different GC release rates from captive salamanders, and that the
393 direction of the difference (i.e., higher, or lower rates) differed between species (Gabor et al.,
394 2016). Therefore, we suggest that researchers assess whether captive-bred vertebrates are good
395 models for their wild counterparts before extensive tests of GC responses to ECs are undertaken.
396 In the interim, our results suggest that this might be a safe practice for fish.

397 *4.3 Baseline versus challenge-induced measures*

398 We found that baseline and challenge-induced GC measures provide similar information
399 regarding the effect of EC exposure on captive-bred fish GC responses. However, baseline GC
400 levels and challenge-induced GC levels have very different physiological effects on the organism
401 (Romero 2004; Sapolsky et al., 2000). Vertebrates maintain baseline levels of GCs that follow
402 circadian, seasonal, and ontogenetic cycles (Romero, 2002; Romero et al., 2008; Wada, 2008;
403 Windle et al., 1998). Baseline levels of GCs are often associated with the permissive actions of
404 GCs (Romero, 2004; Sapolsky et al., 2000). When a vertebrate organism experiences an acute
405 stressor (e.g., predator encounter) further increases of GCs beyond baseline levels occur, which
406 are described as challenge-induced GC levels. At challenge-induced concentrations, GCs bind to
407 glucocorticoid receptors (GRs); GRs function to mediate the stimulatory, suppressive, and
408 preparative effects of GCs (Romero, 2004; Sapolsky et al., 2000). More traditional physiological
409 responses associated with the stress response such as immunosuppression and increased energy
410 mobilization are associated with challenge-induced GC levels and GR binding (Romero, 2004;
411 Sapolsky et al., 2000). Therefore, by measuring only baseline or challenge-induced GC levels,
412 researchers may not be getting a complete assessment of the effects of stressors, such as ECs, on
413 the vertebrate stress response. To provide a comprehensive understanding of GC responses
414 researchers may want to explore both baseline and challenge-induced measures in future studies.

Such comparisons between baseline and challenge-induced responses might seem artificial, but they give an indication of which arm of the HPI axis might be expected to show greater response to EC exposure. More work is clearly needed as we were only able to investigate six classes of ECs in this study and solely on fish GC responses.

4.4 Duration of exposure

Duration of exposure and concentration of the EC was expected to influence the direction and magnitude of fish GC responses across contaminant classes. Duration of exposure seems to be an important moderator variable for fish GC response to EC exposure. Two of our models show statistical support for longer duration of exposure being correlated to decreased GC responses in fish exposed to ECs (captive-bred vs wild-caught, baseline vs challenge-induced, where we had sufficient representation across a reduced set of contaminant classes). The models that do not have a statistically supported posterior distribution for duration of exposure still show a general negative trend between duration of exposure and effect size. This GC response to duration of EC exposure could be due to acclimation to a chronic stressor (the particular EC) over time. Fish exposed to a chronic stressor may show an initial increase in GCs but may not respond at the same level over prolonged exposure to the stressor, with GC levels returning to the same level as unexposed fish, even with the stressor still present (Pickering and Stewart, 1984; Pottinger and Pickering, 1992). Acclimation to a chronic stressor can have consequences for the vertebrate organism. Acclimation can lead to the facilitation of enhanced responses to subsequent stressors (Walker et al., 2020).

Another phenomenon that can occur with exposure to a chronic stressor is fish no longer being able to mount a response to novel stressors. Madaro et al., (2015) chronically exposed Atlantic salmon (*Salmo salar*) to stressors over a 3-week period, after the three weeks, salmon were exposed to a final novel stressor. Chronically stressed fish had significantly lower cortisol

levels compared to controls, showing an inability to mount an appropriate GC response to the novel stressor (Madaro et al., 2015). The study showed that in the pituitary gland of chronically stressed fish had increased expression of genes involved with negative feedback regulation (Madaro et al., 2015). Furthermore, a general downregulation of important receptors, hormones, and enzymes involved in the cortisol stress response of fish was noted after exposure of chronically stressed fish to a novel stressor (Madaro et al., 2015). Therefore, exposure to a chronic stressor may result in an inability of fish to mount an appropriate cortisol response to novel stressors. Therefore, we believe that further investigation into how chronic exposure to ECs influences the fish response to novel stressors is warranted. Additionally, time-series studies examining the GC response of vertebrates exposed to ECs may help to further identify critical time periods of exposure that could lead to GC thresholds (low or high) being reached. Breaching of thresholds might lead further to physiological consequences such as increased disease susceptibility.

4.5 Study Limitations

When conducting a meta-analysis there is always the possibility that relevant studies have been missed during the literature search and eligibility screening stages. However, the dataset likely still offers a representative and non-biased sampled of effect sizes. We did not detect publication bias in our dataset based on established statistical methods, and that our sample size was large with 173 calculated effect sizes. Another limitation of the study is the high number of ECs included in the dataset. We grouped ECs into 11 functional categories, we had multiple datapoints within each EC category. However, within these 11 categories, we had a total of 42 different types of ECs in the dataset based on 44 studies, which in many cases makes us unable to evaluate the effect of the same EC in two or more species of fish. Future studies focusing on specific ECs with widespread replication may help to evaluate our findings.

4.6 Future directions

Our first recommendation for future research is to investigate more fully whether increased duration of exposure to ECs affects the GC response of fish or other vertebrates. Chronic activation of the HPA/HPI axis through prolonged exposure to ECs may result in acclimation to a stressor or in the inability of fish to mount an appropriate stress response to a novel stressor. Future research could help our understanding of potential consequences of EC exposure by examining the effects of novel stressors on the fish GC response after chronic exposure to ECs. Additionally, time-series studies examining the GC response of vertebrates exposed to ECs would be helpful to identify critical time periods of exposure to ECs that may result in significant changes to GC levels.

We also suggest broadening the research of the effects of ECs on the GC response to different vertebrate classes. Our literature search revealed a paucity of research on this topic for amphibian, avian, reptilian, and mammalian species. Broadening the research to have greater representation from less-studied vertebrate classes would allow researchers to explore the effects of EC exposure in both aquatic and terrestrial environments and to test whether particular taxa are more vulnerable to particular ECs.

Related to the second point, we recommend broader research on emerging contaminants such as plastics, per- and polyfluorinated alkyl substances, and salts. The repeated testing of commonly detected ECs such as pesticides and heavy metals is important for assessing reliability of results. However, data are lacking on the sublethal effects of other ECs on fish and wildlife stress metrics, such as GC responses. Knowing the extent to which different ECs influence sub-lethal endpoints such as GC concentrations will aid in understanding how different contaminant groups influence vertebrate health and fitness. This knowledge may aid in making appropriate policy and licensing recommendation around the use of certain compounds.

Finally, we recommend greater consideration of the impact of multiple potential stressors on GC responses. Here we report increases in GC levels in response to ECs, while a previous review examining the impacts of parasite infection on the vertebrate GC response similarly found an overall increase in GC levels for infected hosts (O'Dwyer et al., 2020). A multi-factor approach will lead to a better understanding of the impact of wide-ranging environmental factors on GC levels in vertebrates and the possible synergistic effects they may be having.

5. Conclusions

Exposure to ECs increases the baseline GC levels of captive-bred fish compared to unexposed captive-bred fish, however effect sizes were highly heterogeneous suggesting other moderator variables are important. With respect to contaminant class, pharmaceutical compounds consistently raised baseline GC levels of EC exposed captive-bred fish on average 2.5X above those of unexposed fish. Captive-bred fish exhibited similar GC responses to EC exposure as wild-caught fish suggesting that studies using captive-bred fish may be used to make inferences about ecotoxicity tests involving GC responses for their wild counterparts. We also found no difference between baseline and challenge induced GC measures taken from fish exposed to ECs, but we still encourage researchers to consider measuring both baseline and challenge-induced GC levels to provide a comprehensive understanding of these sublethal responses. Duration of exposure was often negatively correlated to baseline GC levels in exposed fish; chronic exposure to ECs may result in HPI axis acclimation or the inability of fish to mount an appropriate stress response which may have consequences for fish exposure to novel stressors. Such time effects might drown out consistent effects of other moderators such as contaminant class. Finally, we recommend that future research incorporates a broader array of contaminant types and vertebrate classes, and that detailed research be undertaken on mechanisms of HPA/HPI axis effects due to particular ECs.

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