Glucocorticoids in fish eggs: causes of variation and effects on offspring phenotype

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Running head: Effects of egg glucocorticoids on the progeny of fishes

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What Is Already Known: Among oviparous taxa, maternally-derived egg glucocorticoids influence offspring phenotype. Elevated levels of egg glucocorticoids are hypothesized to be signals of maternal/environmental stress, resulting in altered offspring phenotype. Experimental manipulations of egg glucocorticoids provide opportunities to test this hypothesis.

What This Study Adds: This review brings together empirical data on the phenotypic and fitness effects of egg glucocorticoids on the offspring of fishes. This review highlights how effects of natural and maternal stressor-induced variation in egg glucocorticoids on offspring are notably varied, and attempts to explain this variation within methodological, evolutionary and/or ecological contexts. This review provides directions of future research to advance our understanding of the intergenerational function of egg glucocorticoids in fishes.

Abstract

Wild and captive vertebrates face multiple stressors which all have the potential to induce chronic maternal stress (i.e., sustained, elevated plasma glucocorticoids) and thus, elevations in embryo exposure to maternally-derived glucocorticoids. In oviparous taxa, such as fish, maternally-derived glucocorticoids in eggs are known for their capacity to shape offspring phenotype. Using a variety of methodologies, scientists have quantified maternally-derived levels of egg cortisol, the primary glucocorticoid in fishes, and examined the cascading effects of egg cortisol on progeny phenotype. Here we summarize and interpret the current state of knowledge on egg cortisol in fishes, and the relationships linking maternal stress/state to egg cortisol and offspring phenotype/fitness. Considerable variation in levels of egg cortisol exists across species and among females within a species; this variation is hypothesized to be due to interspecific differences in reproductive life history and intraspecific differences in female condition. Outcomes of experimental studies manipulating egg cortisol vary both inter- and intra-specifically. Moreover, while exogenous elevation of egg cortisol (as a proxy for maternal stress) induces phenotypic changes commonly considered to be maladaptive (e.g., smaller offspring size), emerging work in other taxa suggests there can be positive effects on fitness when the offspring's environment is taken into account. Investigation into i) mechanisms by which egg cortisol elicits phenotypic change in offspring (e.g., epigenetics), ii) maternal and offspring buffering capacity of cortisol and, iii) factors driving natural variation in egg cortisol and how this variation affects offspring phenotype and fitness, are all germane to discussions on egg glucocorticoids as signals of maternal stress.

1. Introduction

Animals in the wild regularly encounter multiple stressors and have evolved adaptations to cope with ecological challenges such as perturbations in the abiotic environment (e.g., fire, flooding), predators, resource limitation, and intra- and interspecific competition (Boonstra 2013). One of the coping mechanisms encompassed within the vertebrate stress response is the activation of the HPA/-I axis (i.e., hypothalamic–pituitary–adrenal [HPA] axis in mammals, birds, reptiles (see Fig. 1 in Boonstra 2013); HP-interrenal [HPI] axis in fishes, Wendelaar Bonga 1997, [Fig. 1*a*]), resulting in the production of glucocorticoids (GCs). The elevation of circulating GCs in response to an environmental stressor is considered to be adaptive, initiating physiological and behavioural changes that function to promote survival (Wingfield et al. 1998; Sapolsky et al. 2000; McEwen and Wingfield 2003). Accordingly, GCs are the most pervasive, physiological indicator that an animal has been exposed to a stressor (Cooke and O'Connor 2010). However, compounded with ecological stressors, wildlife now encounter human-induced rapid environmental change (HIREC; e.g., habitat degradation, climatic change, Sih et al. 2011), and animals must now cope with novel stressors and unique combinations of stressors not previously encountered in their evolutionary history (Sih et al. 2011). Under these circumstances, energetically costly processes, such as reproduction, may be sacrificed for increased chances of survival, with these trade-offs being mediated by GCs (Ricklefs and Wikelski 2002). Such trade-offs affect not only to the organism itself, but also in increased exposure of its offspring to maternal GCs and intergenerational phenotypic programming (Love et al. 2013).

Populations of fishes regularly encounter stressors and challenging conditions that can elevate circulating levels of the GC, cortisol (e.g., predation threat, Rehnberg and Schreck 1987; intraspecific competition, Ejike and Schreck 1980). Stressor exposure activates the HPI axis resulting in a biochemical cascade that initiates at the hypothalamus and concludes in the interrenal cells of the head kidney with the synthesis and elevation of cortisol (Fig. 1*a*). Elevations in circulating cortisol are hypothesized to also elevate egg cortisol in reproductive females (Fig. 1*c*), with the potential for downstream effects on offspring (Fig. 1*b*). Marine and freshwater fishes now additionally face novel types of anthropogenic stressors that are outside their evolutionary history, including climate change-mediated elevations in water temperatures (e.g., Chadwick et al. 2015), interactions with fisheries (e.g., Marçalo et al. 2009), and deteriorated habitats due to human activities (e.g., sediment loading, Awata et al. 2011). Domesticated and farmed fishes are also subjected to stressors associated with husbandry and aquaculture (e.g., handling, confinement, and transport, Barton & Iwama 1991; noise, Anderson et al. 2011). These stressors can all elevate circulating levels of maternal cortisol, which may result in altered offspring phenotypes via elevations in egg cortisol, calling into question the ability of the next generation to cope with its environment. As such, determining the evolutionary role of variation in maternally-derived GCs has become a topic of great interest in the field of integrative ecology (Sheriff and Love 2013). For example, information on variation in egg GCs has the potential to be translated into metrics of broodstock health and production quality for aquaculture and stock-enhancement initiatives. Moreover, measurements of GCs in wild animals have the potential to act as indicators of population stress for conservation practitioners (Madliger and Love 2014; Sopinka et al. 2015*a*). Despite research spanning almost two decades, patterns of cortisol investment in fish eggs have yet to be comprehensively

95 synthesized, and integrated with prevailing assumptions of intergenerational mechanisms of
96 stress.

97

98 Here we synthesize the current knowledge base on the relationships among maternal
99 stress, maternally-derived egg GCs, and offspring phenotype in fishes. First, we briefly review
100 research regarding maternal GCs and oviparity in avian systems as it can help guide research
101 conducted in fishes (Section 2). Next, studies of maternal stress and egg GCs in fishes are
102 reviewed including detailed descriptions of the mechanisms of action of egg GCs (Section 3.1)
103 and the metabolism of egg GCs pre- and post-fertilization (Section 3.2). We then summarize and
104 interpret literature on cortisol concentrations in fish eggs, focusing on the cascading effects of
105 natural variation in egg cortisol (Section 4) and experimentally-induced variation in egg cortisol
106 (Section 5) on offspring phenotype and fitness. Based on this information we suggest pertinent
107 avenues of future research (Section 6) and provide conclusions (Section 7).

108

109 **2. Maternal GCs and oviparity: what do we know from birds?**

110 Considerable research within the biomedical realm, showing that stressor-induced and
111 exogenously manipulated maternal GCs are robustly linked with maladaptive offspring
112 phenotypes in humans and rodents (reviewed by Seckl 2004; Cottrell and Seckl 2009), has
113 guided predictions regarding the effects of maternal GCs on offspring in non-mammalian taxa,
114 such as birds. After mammals, intergenerational effects of stress are most extensively studied in
115 birds, whereby maternal GCs are deposited into eggs (reviewed in Henriksen et al. 2011). The
116 concentration of egg GCs can vary by timing of breeding and laying order (Love et al. 2008),

and these patterns can additionally differ by life-history strategy (Love et al. 2009) and by breeding selection regimes (high and low plasma GC response, Hayward et al. 2005; fast and slow growth, Ahmed et al. 2013). Importantly, maternal stressor exposure (Okuliarová et al. 2010) and experimental manipulation of maternal plasma GCs (Hayward and Wingfield 2004; Love et al. 2005) both result in eggs with elevated levels of GCs compared to unexposed/non-manipulated females. A number of studies have then directly manipulated egg GCs (as a proxy for maternal stress) to examine effects on offspring phenotype. For some avian studies, results align with mammalian models of maternal stress; prenatal (i.e., in the egg) exposure to elevated GCs produces offspring with phenotypes commonly interpreted as maladaptive (e.g., reduced body size/feather growth, Saino et al. (2005); reduced competitive ability, Janczak et al. (2006); reduced begging intensity, Rubolini et al. (2005)). However, as Henriksen et al. (2011) conclude, there is notable variation in the directionality of effects egg GCs have on offspring phenotype (e.g., increased body size, Tilgar et al. (2016); enhanced flight performance, Chin et al. (2009); increased begging intensity, Love and Williams (2008)). In turn, this growing body of literature in avian systems has supported and enhanced the interpretation of the effects of maternal GCs on offspring fitness in other oviparous taxa, including fishes.

3. Egg GCs in fishes

Similar to avian species, GCs (cortisol) in eggs of fishes are maternally derived (Table 1) and necessary for proper offspring development (Nesan and Vijayan 2013*a*,*b*). Cortisol, a lipophilic steroid, is reported to be incorporated into eggs during vitellogenesis, a late stage of oogenesis whereby glycolipoproteins (e.g., vitellogenins (Vtg)) are taken up by the follicle and

139 processed into yolk (Fig. 2, see Brooks et al. (1997), Jalabert (2005), and Lubzens et al. (2010)
140 for further details on teleost oogenesis). Hormones enter the vitellogenic follicle by diffusion
141 along a concentration gradient (Tagawa et al. 2000), or possibly via co-entry with Vtg (Brooks et
142 al. 1997), and accumulate in the yolk (Fig. 2). *In vitro* incubation of follicles in media with and
143 without radio-labelled cortisol also suggests bidirectional movement of cortisol between follicles
144 and maternal circulation (Tagawa et al. 2000; Fig. 2).

145 While the biochemical and physiological relationships among cortisol, the stress response
146 (HPI axis, Fig. 1*a*), and reproductive parameters (e.g., circulating levels of sex steroids, egg size,
147 fecundity, fertilization success) in fishes have been extensively addressed (e.g., Iwama et al.
148 1997, Wendelaar Bonga 1997, Mommsen et al. 1999, Milla et al. 2009), studies on the
149 intergenerational effects of stressor-induced GCs in fish are not as abundant. Pioneering studies
150 by Campbell and colleagues found that female salmonids that were chronically stressor-exposed
151 had elevated plasma cortisol levels, eggs of smaller size, and reduced survival of embryonic
152 offspring (Campbell et al. 1992, 1994). Schreck et al. (2001) were among the first to synthesize
153 known maternal effects of stress in fishes at a time when there was still limited knowledge of the
154 intergenerational effects of stress and only a handful of new studies (e.g., Contreras Sánchez et
155 al. 1996, 1998; Stratholt et al. 1997; McCormick 1998). These new studies did however provide
156 important insight into a potential mechanism underlying maternal stressor-induced offspring
157 change, namely elevated egg cortisol concentrations. Stressor-induced elevations in maternal
158 plasma cortisol (and proxies thereof *via* intraperitoneal injection of cortisol, McCormick 1998)
159 can result in hypercortisolism of fish eggs (Fig. 1*c*; Stratholt et al. 1997). Since then, research
160 across species has demonstrated how elevations in egg cortisol shape offspring phenotype (Table
161 2).

3.1 Mechanisms of action

When relationships are detected between egg cortisol and offspring phenotype, how do these hormonally-mediated phenotypes manifest? In adult fishes, cortisol binds to tissue-specific GC receptors (GRs) and this intracellular ligand-receptor complex moves to a cell's nucleus. In the nucleus, the ligand-receptor complex binds to glucocorticoid response elements on DNA and induces transcription (Bury and Sturm 2007). Maternal transcripts for GRs are detected in newly fertilized zebrafish (*Danio rerio*) eggs and extensive work on this model species has revealed the mechanistic actions of maternally-derived cortisol and GR transcripts in mediating offspring development (e.g., regulating development of the stress axis; Nesan and Vijayan 2013*a*,*b*). Pikulkaew et al. (2011) postulated that, in zebrafish, binding of maternally-derived cortisol to GRs, translated from maternal GR transcripts, was possible shortly after fertilization (Fig. 1*b*). Recently, Nesan and Vijayan (2016) used microinjection of cortisol antibody to sequester maternally-derived cortisol from single-cell zebrafish embryos and found that the cortisol stress response of embryos 72 hours post-fertilization was heightened (i.e., higher post-stressor whole body cortisol levels compared to control embryos), and transcript abundance of HPI axis genes in embryos 48 hours post-fertilization was altered (see Table 2). The authors concluded that maternal cortisol is integral to the formation of the stress response (Nesan and Vijayan 2016). Following knockdown of GR protein content in zebrafish embryos using morpholino oligonucleotides, Pikulkaew et al. (2011) and Nesan et al. (2012) found that growth, swim bladder and craniofacial development, and survival of larval offspring were altered. In addition, transcript abundance of genes for extraceullar matrix remodeling, bone morphogenesis, and myogenesis and cell proliferation were also altered in larval offspring (Pikulkaew et al. 2011;

185 Nesan et al. 2012). Thus, maternal cortisol is thought to affect offspring development via GR
186 signaling effects on transcript abundance (Pikulkaew et al. 2011; Nesan et al. 2012; Nesan and
187 Vijayan 2013*a*,*b*; Fig. 1*b*). Furthermore, knockdown of GR protein content also alters transcript
188 abundance and behavioural phenotypes in adult zebrafish (Wilson et al. 2015; 2016). Using
189 mutant zebrafish (gr^{s357}) with non-functional GRs, Griffiths et al. (2012) and Ziv et al. (2013)
190 found that the startle response, locomotor activity, and exploratory and social behaviours are
191 altered compared to control fish. Again, these whole-organism changes suggest that effects of
192 maternally-derived cortisol on offspring phenotype are mediated by GR signaling (Fig. 1*b*). It is
193 noted that zebrafish have only a single GR gene. Indeed, Alsop and Vijayan (2009) posit whether
194 other fishes with multiple GR genes (e.g., rainbow trout *Oncorhynchus mykiss*) "have different
195 mechanisms or abilities to cope with stressors" compared to zebrafish. For example, in rainbow
196 trout, offspring transcription levels of genes associated with GRs, nuclear receptor superfamily
197 proteins and insulin-like growth factor, can be altered when ovarian follicles/eggs are treated
198 with the GR antagonist Mifepristone/RU486 (Li et al. 2012*b*; Ferris et al. 2015). Continued
199 extension of the genomic and physiological tools available for use in zebrafish to other teleost
200 species is warranted.

201 Cortisol-mediated epigenetic changes are also thought to account for changes in offspring
202 phenotype (Li et al. 2010; Pikulkaew et al. 2011; Nesan and Vijayan 2013*a*). Drawing largely on
203 what is known from mammalian literature, Love et al. (2013) and Li and Leatherland (2013)
204 highlight epigenetic programming as a viable mechanism whereby maternal stress/GCs can
205 cause phenotypic change in the offspring of oviparous taxa. Again, GR signaling is implicated in
206 this mechanistic pathway as GRs are subject to maternally-mediated epigenetic programming in
207 mammals (Weaver 2009). In embryonic threespined stickleback (*Gasterosteus aculeatus*),

208 Mommer and Bell (2014) found that variation in the expression of DNA methyltransferase and
209 histone genes depended on whether their mothers were stressor-exposed (i.e., threat of predation)
210 or left undisturbed. In birds, *in ovo* injection of GCs increased DNA methylation of the
211 hypothalamic GR gene promoter (Ahmed et al. 2014). The interactions among maternal stress,
212 egg hormones, and epigenetic regulation are complex, and efforts to further understand the
213 proximate mechanisms of action of egg cortisol will be relevant in predicting how early-life
214 effects contribute to phenotypic change of offspring.

215

216 ### *3.2 Pre- and post-fertilization metabolism of egg GCs*

217 In fishes, *in vitro* and *in vivo* studies indicate that developing ovarian follicles, fertilized
218 eggs, and pre-hatch embryos are capable of metabolizing steroid hormones, including cortisol.
219 These findings match what is known in other oviparous taxa such as birds (Vassallo et al. 2014)
220 and reptiles (Paitz and Bowden 2013). Based on observations that maternal plasma cortisol levels
221 are significantly higher than those measured in eggs, and therefore indicative of blood-egg
222 buffering, Schreck et al. (2001) proposed a "progeny-protecting" system. Along with attenuation
223 of maternal HPI activity as sexual maturation progresses (i.e., attenuated plasma cortisol
224 response to a stressor), and corticosteroid binding proteins restricting transfer of free cortisol
225 from maternal circulation to eggs, Schreck et al. (2001) also hypothesized that enzymes capable
226 of metabolizing cortisol, such as 11β-hydroxysteroid dehydrogenase 2 (11βHSD2) which
227 metabolizes GCs in the mammalian placenta (Benediktsson et al. 1997), are present in ovarian
228 follicles. Indeed, Tagawa et al. (2000) detected metabolism of cortisol (to cortisone, the
229 biologically inactive GC in fishes, Bury and Strum 2007) in the thecal/granulosa layer of tilapia

(*Oreochromis mossambicus*) follicles following *in vitro* incubation with radio-labelled cortisol. More recently, Li et al. (2012*a*, 2014) showed that rainbow trout ovarian follicles metabolize cortisol to cortisone as well as cortisol- and cortisone sulphates (Fig. 1*c*). Li et al. (2012, 2014) inferred this metabolism to indicate presence and activity of 11βHSD2 and glucocorticoid sulphotransferase (GST; sulfonation as a buffering pathway of maternally-derived steroid hormones in oviparous taxa is reviewed by Paitz and Bowden (2013)) (Fig 1*c*). Indeed, Kusakabe et al. (2003) detected 11βHSD2 transcripts in the thecal and granulosa cells of rainbow trout ovaries, and found that transcript abundance increased throughout sexual maturation/vitellogenesis. Recently, Faught et al. (2016) found 11βHSD2 transcript abundance in zebrafish follicles also increased following incubation with cortisol *in vitro*, suggesting, in response to maternal stress, there is upregulation of enzymes in ovaries that reduce cortisol levels in eggs. There are therefore multiple biochemical pathways to examine as potential pre-fertilization mechanisms that control excess cortisol in fish eggs.

Regarding the potential for post-fertilization buffering, Leatherland et al. (2010) focused on the interconnectedness of cortisol and the HPI and HP-ovary (HPO) axis, concluding that egg cortisol has "a relatively minor influence on early ontogeny" and that this may be due in part to the "ability of embryos to metabolize cortisol to form steroids that have a low biological activity." In fishes, the onset of endogenous cortisol production in response to a stressor is observed pre-hatch (Stouthart et al. 1998), at hatch (Barry et al. 1995; Jentoft et al. 2002), and at first feeding (Alsop and Vijayan 2008). Other components of the HPI axis (e.g., upregulation of genes associated with cortisol production) can be responsive to a stressor prior to when differences in cortisol are detected (Fuzzen et al. 2011). Yet, in much earlier stages of progeny development, steroid hormone levels are dynamic. Across species, newly fertilized eggs are able

to clear maternally-derived cortisol as indicated by significant reductions in cortisol concentrations within 24 hours post-fertilization (e.g., coho salmon *Oncorhynchus kisutch*, Sopinka et al. 2015*b*; Japanese flounder *Paralichthys oliwceus*, de Jesus et al. 1991; silver carp *Hypophthalmichthys molitrix*, Kauser et al. 2013; white sturgeon *Acipenser transmontanus*, Simontacchi et al. 2009; zebrafish, Nesan and Vijayan 2012). Li et al. (2012*a*) also found conversion of cortisol to other metabolites in ovulated oocytes and embryos 25-58 days post-fertilization (dpf) (but see Paitz et al. 2016 for absence of metabolism in embryonic threespined sticklebacks). However, the extent of metabolism was less than that observed in ovarian follicles (Li et al. 2012*a*). Recently, Paitz et al. (2016) found evidence for excretion of cortisol from newly fertilized threespined stickleback eggs via ABC (ATP-binding cassette) transporters (Fig. 1*c*), which are transmembrane transport proteins associated with uptake of xenobiotics in fishes (Luckenbach et al. 2014). There remains much to be gleaned regarding post-fertilization buffering mechanisms in fishes.

The consensus is that ovarian follicles, eggs, and embryos are not, "passive recipients of maternal steroids" (Vassallo et al. 2014) nor "passive responders to the levels of steroids present in eggs" (Paitz and Bowden 2013). Moore and Johnston (2008) address numerous questions regarding the deposition, regulation, and metabolism of yolk steroids in oviparous taxa. These notions have implications for experimental design (i.e., sampling time points) and data interpretation. Although cortisol is thought to accumulate in egg yolk, the capacity for follicles to metabolize cortisol and for newly fertilized eggs to transport cortisol out of the embryo demonstrates that if concentrations are only measured at one life stage (e.g., unfertilized eggs), the concentrations represent a snapshot in time of a fluctuating hormone. Further, GR density and affinity in species with multiple GRs, as well as the percentage of bound versus unbound

276 cortisol in maternal circulation, are apt to influence egg cortisol-mediated effects. These notions
277 can have implications with regard to the predicted roles of egg cortisol in fishes.

278

279 **4. Natural variation in egg cortisol**

280 The presence of inter-individual variation in egg GCs before fertilization, and its
281 downstream potential to influence offspring phenotype and fitness following fertilization, sets
282 the stage for natural selection to act on mothers and offspring in response to environmental
283 variation (Love and Williams 2008). We focus here on studies that report variation in
284 concentrations of maternally-derived cortisol in ovarian tissue, unfertilized eggs, and eggs
285 sampled at fertilization.

286 Maternally-derived cortisol is detected in unfertilized and newly fertilized eggs of many
287 fish species (Table 1). Within a species, variation in egg cortisol content is observed across
288 studies; egg cortisol levels in rainbow trout range from 5 to 60 ng g^{-1} (Table 1). This type of
289 variation is potentially driven by differences in genetic or environmental factors associated with
290 different suppliers/strains of females or laboratory rearing practices, respectively. Although not
291 frequently reported in the literature, the absolute ranges of egg cortisol levels detected among
292 females within a given study can be quite substantial in both freshwater (brown trout, *Salmo*
293 *trutta*, 3.22 to 122.47 ng g^{-1}, Burton et al. 2011) and marine species (damselfish, *Pomacentrus*
294 *amboinensis*, 0.3-76.0 ng g^{-1}, McCormick 1998).

295

296 ***4.1 Drivers of variation***

297 Examining the environmental/ecological drivers of variation in maternal GCs during
298 reproduction, and therefore the potential for maternally-derived GCs to act as maternal signals
299 (Nesan and Vijayan 2013a) linking a mother's and offspring's environments, has become an
300 important focus in studies of maternal stress (Love et al. 2013; Crossin et al. 2016). For a given
301 study, there are several questions to consider when hypothesizing what factors may be
302 influencing inter-female variation in egg cortisol. (1) Does population-specific life history dictate
303 egg cortisol content, as has been suggested in other taxa (e.g., Love et al 2009)? Egg cortisol
304 levels vary among geographically-distinct populations of Chinook salmon (*Oncorhynchus*
305 *tshawytscha*) that differ in the distance adults migrate from the ocean to reach freshwater
306 spawning grounds (Table 1); egg cortisol levels are higher in females that swim a longer distance
307 to reach spawning areas. In Pacific salmon, egg number and size appear to be selected for
308 population-specific migration distances (Beacham and Murrary 1993). Egg cortisol levels may
309 be another trait selected for based on population-specific environmental conditions; however,
310 without a greater understanding of how natural variation in egg cortisol influences offspring
311 phenotype, this explanation lacks an evolutionary basis. Egg cortisol content also varies between
312 farmed and wild stocks of Chinook salmon (Table 1). It is well known that numerous traits vary
313 between domesticated and wild salmonids (Weber and Fausch 2003). Differences in egg cortisol
314 between farmed and wild stocks are likely the outcome of selection regimes, but the traits
315 targeted for selection that are linked with egg cortisol are not presently known. On a finer spatial
316 scale, cortisol levels detected in laid clutch masses did not significantly vary between benthic
317 and limnetic populations of threespined stickleback (Foster et al. 2015). The ecotypes are
318 distinguished by their foraging mode and one might predict that a reproductive trait, such as egg
319 cortisol, would not vary between populations. To address these hypotheses, studies quantifying

population-specific variation in egg cortisol and relating this variation to variation in maternal/offspring fitness are needed.

(2) For females that spawn multiple times a year, is egg cortisol content contingent on clutch order? Sampath-Kumar et al. (1995) found that egg cortisol concentrations in newly fertilized Asian seabass (*Lates calcarifer*) eggs varied depending on when females were spawned. Females spawned in January and February had mean egg cortisol concentrations of 1.20 ng g^{-1} and 0.62 ng g^{-1}, respectively. Females spawned in March of the same year had mean egg cortisol concentrations of 2.20 ng g^{-1}. These differences in egg cortisol of ~1 to 1.5 ng g^{-1} could elicit variation in offspring phenotype given that differences in egg cortisol of ~3 ng g^{-1} elicit changes in offspring HPI function (Auperin and Geslin 2008). The farmed Asian seabass used by Sampath-Kumar et al. (1995) spawn year round. Egg cortisol content may vary temporally because of fluctuations in female condition but what/how seasonal influences are modulating female condition is not known.

(3) Is egg cortisol content driven by habitat choice? While there is relatively little information currently available to answer this question, McCormick (1998) did not find that ovarian cortisol varied among damselfish nesting at different locations in a coral reef. The author does not specify how the locations differ other than spatially, but a lack of difference in egg cortisol suggests the reef (and maternal) environment in this study was relatively uniform (e.g., equal abundance of resources, competitors, predators among locations).

(4) Does maternal social status or stress coping style regulate egg cortisol content? Egg cortisol content did not differ between dominant and subordinate zebrafish (Jeffrey and Gilmour 2016). This was contrary to the author's predictions given that subordinate fish have chronically

342 elevated plasma cortisol (Sloman et al. 2001), thus, one would expect elevation of egg cortisol to
343 align with elevated maternal cortisol (Stratholt et al. 1997). Rainbow trout bred for high- and
344 low-responsiveness to an acute stressor yield offspring that differ with regard to yolk sac size
345 and timing of emergence from spawning gravel (Andersson et al. 2011; 2013). However, egg
346 cortisol content does not differ between the strains of rainbow trout (Andersson et al. 2011),
347 suggesting that egg cortisol does not appear to be the mechanism coupling stress coping style
348 and other traits across generations. In contrast, Atlantic halibut (*Hippoglossus hippoglossus*)
349 more resistant to handling (i.e., "unrested" and requiring "force to keep the fish on the table until
350 it settled") had lower concentrations of cortisol in embryos collected 1 dpf (Skaalsvik et al.
351 2015). This finding suggests that maternal stress coping style could be linked with egg cortisol,
352 although comparison of cortisol levels in unfertilized eggs (*versus* recently fertilized eggs) is
353 required. Overall, the scope of variation among females described above suggests that allocation
354 of egg cortisol has the potential to confer some influence on maternal fitness.

355

356 ***4.2 Relationships between egg cortisol variation and offspring phenotype/fitness***

357 Ascertaining the impacts of variation in maternally-derived GCs on offspring phenotype,
358 and fitness, using correlation data is often difficult to determine given that underlying
359 costs/benefits of exposure may be hidden without experimental manipulation (Crossin et al.
360 2016). As such, a dearth of knowledge still remains to be uncovered regarding the connections
361 between inter-female variation in egg cortisol levels and offspring phenotype/fitness in fishes,
362 especially since following egg cortisol analyses, embryos are not always reared long-term. In a
363 coral reef damselfish, across females, higher concentrations of ovarian cortisol were associated

with shorter larvae (McCormick 1998). A similar trend appeared to emerge in sockeye salmon (*Oncorhynchus nerka*), whereby fry body condition decreased with increasing egg cortisol concentration (Sopinka et al. 2014). This same study did not find any correlation between egg cortisol and fertilization success or embryonic survival (Sopinka et al. 2014). From studies using embryos ~1 dpf, and noting that teleost embryos metabolize steroid hormones (Section 3.2), indication of how variation in cortisol may govern variation in early development of offspring can be gleaned. Occurrence of yolk-sac edema increased with increased embryo cortisol content (1 dpf) in Atlantic halibut, although embryo cortisol content did not directly correlate with other parameters including fertilization success or larval size (Skaalsvik et al. 2015). In smallmouth bass (*Micropterus dolomieu*), eggs were collected from nests, and eggs with higher cortisol had lower hatching success in the laboratory (Gingerich and Suski 2011). Cortisol levels measured in eyed embryos of masu salmon (*Oncorhynchus masou*) were negatively correlated with survival to the eyed life stage (Mingist et al. 2007). Natural variation in egg cortisol thus has the potential to shape offspring phenotype/fitness, yet research exploring the extent to which natural variation in egg cortisol dictates these offspring parameters later in development is limited. Long-term rearing of free-swimming offspring can be logistically challenging and require utilization of a marking system (e.g., PIT or elastomer tagging) if offspring are to be segregated by maternal identity/egg cortisol content. Furthermore, given that aquatic ecosystems are subject to HIREC, this naturally-occurring variation may be subject to novel selective pressures and be accompanied by new modifications to offspring phenotype.

5. Experimental manipulation of egg cortisol

5.1 Methodologies

Manipulation of egg GCs directly via exposure of eggs, or indirectly via manipulating the female, enables researchers to separate correlation from causation and illuminate the evolutionary significance of variation in maternally-derived GCs (Meylan et al. 2012). There are several methods used to manipulate egg cortisol levels *in vivo*. Maternal environments can be altered and egg cortisol quantified (e.g., exposure of females to a physical stressor: Stratholt et al. 1997; Sopinka et al. 2014; Ghio et al. In press; exposure of females to conspecific competition: McCormick 2006, 2009; Jeffrey and Gilmour 2016; exposure of females to anthropogenic noise: Sierra-Flores et al. 2015). Given that steroids are lipophilic, egg cortisol concentrations can be indirectly manipulated with intraperitoneal injection of cortisol emulsified in cocoa butter or oil (Eriksen et al. 2006). However, caution should be heeded as the injection medium itself (rather than elevated egg cortisol *per se*) can also affect offspring size. Hoogenboom et al. (2011) found that, compared to unmanipulated female brown trout, egg and offspring size were smaller in females that were injected intraperitoneally with sham and cortisol-dosed cocoa butter. For larger-bodied fishes, osmotic pumps implanted into females offer an alternative to intraperitoneal injections (Kleppe et al. 2013). Food pellets soaked in cortisol-laced solutions are a viable option for species too small for surgery (e.g., zebrafish, Faught et al. 2016). Egg cortisol concentrations can be directly manipulated with microinjection of cortisol into eggs (zebrafish, Nesan and Vijayan 2012), bathing of unfertilized eggs in ovarian fluid (brown trout, Sloman 2010), or bathing of eggs at fertilization (coho salmon, Sopinka et al. 2015*b*). Each methodology has advantages and disadvantages (see Gamperl et al. 1994; Sopinka et al. 2015*a*) depending on the species and question of interest, and care should be taken when choosing an appropriate tool to manipulate egg cortisol.

5.2 *Effects of experimentally elevated egg cortisol on offspring phenotype and fitness*

The array of phenotypic traits investigated following egg cortisol treatment is substantive, spanning from genomic to whole-animal responses (Table 2). Collectively, there does not appear to be a consistent manner of change in offspring phenotype/fitness following experimentally elevated egg cortisol (Table 2). For example, effects of egg cortisol treatment on size of fry, a recognized predictor of offspring performance in fishes, are either not reported, not detected, or depend on the dose of egg cortisol treatment (Table 2). Aggression/dominance is both increased (Sloman 2010; Sopinka et al. 2015*b*) and reduced (Burton et al. 2011) in salmonids reared from cortisol-treated eggs. Effects of exogenously elevated egg cortisol on activity levels in rainbow trout offspring vary across ontogeny; at 5 months post-fertilization, offspring from cortisol-treated eggs were more active than offspring from untreated eggs, but there were no differences in activity levels at 2 months post-fertilization (Colson et al. 2015). In Atlantic salmon (*Salmo salar*), effects of elevated egg cortisol on offspring response to a confinement stressor were also dependent on age (Eriksen et al. 2011, 2013). Four months post-hatch offspring reared from cortisol-manipulated females were more active during acute confinement compared to offspring reared from sham females (Eriksen et al. 2013), whereas, 1.5 years post-hatch offspring reared from cortisol-manipulated eggs were more inactive during acute confinement compared to controls (Eriksen et al. 2011). Differences may be due to differences in time of confinement (20 versus 30 minutes) and size of confinement tank (0.5 L versus 1.5 L). On a study-by-study basis, findings can be argued to be beneficial (e.g., dominance, increased offspring size) or detrimental (e.g., subordinance, decreased offspring size). However, without measuring fitness outcomes of a specific phenotype, phenotype alone cannot be affirmed as adaptive or maladaptive.

Interpretation of the modified trait as adaptive or maladaptive, in conjunction with the hypothesis that elevated egg cortisol acts as a maternal signal to offspring (Nesan and Vijayan 2013*a*), must also consider whether the environment offspring encounter and/or are tested in is matched or mismatched to the maternal environment (see Section 6.2). Effects of elevated egg cortisol on correlates of offspring fitness (e.g., survival to first-feeding) are restricted to early life stages (Table 2). When reported, and with the exception of Li et al. (2010), elevated egg cortisol does not affect embryonic survival. It is possible that egg cortisol is affecting genomic/physiological pathways, but not in a manner that results in embryo death.

5.3 Effects of maternal stressor-induced egg cortisol on offspring phenotype and fitness

Despite an enthusiastic interest in determining how elevations in egg cortisol shape offspring phenotype (Table 2), the evidence to date indicating that maternal stressor exposure modifies egg cortisol levels is ambiguous. There are as many studies that have found differences (e.g., Stratholt et al. 1997; McCormick 2006, 2009; Sierra-Flores et al. 2015) as there are that have not (e.g., Contreras-Sánchez 1996; Mileva et al. 2010; Jeffrey and Gilmour 2016; Sopinka et al. 2014; Ghio et al. In press). There are physiological/biochemical (e.g., maternal and embryonic metabolism of cortisol, see Section 3.2) and logistical (e.g., variation in stressor type and duration) reasons why a study may or may not have detected differences in egg cortisol.

Variation in ovarian development across species could also affect incorporation and quantification of cortisol in the eggs of stressor-exposed females. For synchronous spawning females, with all eggs developing and ovulating at the same time (e.g., salmonids), eggs can be collected from stressor-exposed females at a single time point post-ovulation and variation in

454 cortisol levels can be interpreted as being stressor-induced. However, the effects of timing of
455 stressor application in relation to vitellogenesis and cortisol deposition are not yet clear.
456 Contreras-Sánchez (1996) did not find variation in egg cortisol or embryo viability between
457 undisturbed female rainbow trout and females exposed to a stressor treatment during early
458 vitellogenesis, late vitellogenesis, or during both early and late vitellogenesis. In contrast, in
459 asynchronous fishes such as zebrafish, all eggs of all stages of oogenesis are present in the
460 female and Faught et al. (2016) found temporal patterns in egg cortisol deposition in this species
461 following a 5 day feeding period of cortisol-soaked food pellets. Accordingly, breeding
462 synchronicity/strategy (reviewed in McBride et al. 2015), timing of stressor-exposure, and timing
463 of egg collection are important factors to consider when designing experiments, especially to
464 facilitate comparison across studies.

465 Another common explanatory denominator threaded throughout these studies is the
466 possibility that life history variation could affect if and how a female responds to a specific
467 stressor, and whether these responses would be selected for (Love et al. 2009). Accordingly, it is
468 pertinent that 1) a species' evolutionary history be thought of *a priori*, and 2) experimental egg
469 manipulations are altering cortisol to levels that can be detected in a given species while it is
470 under benign or disturbed conditions (i.e. within a biologically-relevant physiological range)
471 (Crossin et al. 2016). It is important to note that if maternal stressor exposure does not affect egg
472 cortisol, this does not mean that 1) variation in egg cortisol does not have a role in phenotypic
473 trajectories of offspring, or 2) maternal-stressor exposure does not affect phenotypic traits of
474 offspring through other, non-hormonal mechanisms (e.g., epigenetics, Section 3.1).

475

6. Future research directions

Cortisol's presence and functionality in the egg requires further exploration from several perspectives. From a methodological angle, transparent methods that target assessment of cortisol in ovarian follicles/tissue, unfertilized eggs, or newly fertilized eggs will ensure that maternally-derived *versus* endogenous egg cortisol is being assessed and related to offspring phenotype, and allow for meaningful comparison of findings across studies. Combining multiple methodologies within a study is most powerful. For example, Ghio et al. (In press) reared offspring from female brook trout (*Salvelinus fontinalis*) fed cortisol-dosed food or repeatedly handled females, and from eggs bathed in cortisol. There is also scope to investigate how synthetic GCs, such as the pharmaceutical prednisolone that is detected in bodies of water, influence offspring phenotype (McNeil et al. 2016). Other areas of future research include: 1) conducting studies that adequately capture intra- and inter-specific variation, 2) rearing and testing offspring in environments that do and do not match the maternal environment, and 3) assessing carryover effects on adult offspring phenotype/fitness.

6.1 Quantifying multiple levels of variation

Our current understanding of how variation in egg cortisol within a female (e.g., position of egg in the ovary) and among females correlates with offspring phenotypes remains limited, primarily due to low samples sizes and other logistical constraints (e.g., difficulty of rearing maternal lines separately). In brown trout, Suter (2002) found evidence for intra-female variation in egg cortisol depending on position within the ovary (anterior, middle, and posterior). Offspring phenotype of brown trout was later found to also vary according to position within the

498 ovary (Burton et al. 2013*a*), but these data were not directly linked with egg cortisol. Inter-
499 female variation in egg cortisol levels can be sizable for some species (Table 1). Does this inter-
500 female variation account for variation in offspring phenotype? Does this variation relate to
501 variation in maternal condition? In birds, mothers in lower body condition are known to lay eggs
502 with higher concentrations of GCs (Love et al. 2008). Similarly, analyses of female body
503 condition and concentration of cortisol in unfertilized eggs of Pacific salmon show increasing
504 egg cortisol with decreasing maternal condition in wild sockeye salmon (Fig. 3). However, no
505 statistically significant relationships were detected in coho salmon or farmed Chinook salmon
506 (Fig. 3).

507 Unresolved questions concerning how different levels of egg cortisol correlate with
508 offspring phenotype may also be due to concentration thresholds of egg cortisol. As previously
509 mentioned (Section 5.3), the evidence amassed to date does not uphold the prevailing hypothesis
510 that maternal stressor exposure consistently increases egg cortisol content in fishes. Moreover,
511 the relationship between egg cortisol and offspring phenotype may not always be linear (Fig. 4),
512 and is expected to be under evolutionary constraints depending on the predictability and
513 variability of the maternal and offspring environments (Burgess and Marshall 2014). Offspring
514 performance may be optimal within an intermediate range of egg cortisol concentrations and
515 sub-optimal at lower and higher concentrations of egg cortisol outside the range (Fig. 4*a*). For
516 example, Li et al. (2010) found non-linear relationships between egg cortisol and offspring
517 performance in rainbow trout. An egg cortisol treatment of 100 ng mL^{-1} (low dose) resulted in
518 offspring larger than controls (0 ng mL^{-1}) and those reared from eggs treated with 1000 ng mL^{-1}
519 cortisol (high dose), which did not differ in size from controls (Li et al. 2010). This enhanced
520 growth pattern in offspring treated with a low cortisol dose was coupled with amplification of

521 insulin-like growth factor (IGF) transcripts (Li et al. 2010). There could be a negative, linear
522 relationship between offspring performance and concentrations of egg cortisol (Fig. 4*b*; e.g.,
523 McCormick 1998). Offspring performance could be maintained throughout a range of egg
524 cortisol concentrations but decrease at concentrations beyond a specific threshold concentration
525 (Fig. 4*c*; e.g., Eriksen et al. 2006). When possible, implementation of multiple doses of cortisol
526 for egg treatment can help determine concentration-dependent phenotypic effects.

527 Lastly, although not probed in detail in this review, inter-specific variation in egg
528 cortisol levels of fishes is also significant (Table 1). Although the reproductive life history
529 strategies of fishes are remarkably diverse and researchers have both predicted and demonstrated
530 that variation in life histories affects the variability and adaptive potential of egg GCs in other
531 taxa (Love et al. 2009, 2013; Sheriff and Love 2013), little is known about whether egg cortisol
532 is associated with this variation in fishes. For example, among salmonids there are semelparous
533 Pacific salmon and iteroparous trout. One may predict that the buffering capacity of cortisol
534 (Section 3.2) in Pacific salmon would be superior to trout given that Pacific salmon only have
535 one opportunity to reproduce before dying. Species undergoing long-distance migrations for
536 spawning (e.g., Pacific salmon, American shad (*Alosa sapidissima*), Pacific herring (*Clupea*
537 *pallasii*)), and arguably a relatively more stressful reproduction, may deposit more or less
538 cortisol into eggs compared to non-migratory species. Species-specific variation in egg cortisol
539 content may reflect inter-specific variation in early offspring life history and rates of
540 development (e.g., pelagic migration of larval, marine fishes *versus* over-winter rearing of
541 juvenile, freshwater fishes). Continuing to uncover answers to these questions regarding
542 naturally-occurring variation in egg cortisol can bolster support for the hypothesis that GCs act
543 as maternally-derived stress signals (Sheriff and Love 2013). In addition, establishing

associations between maternal condition, egg cortisol, and offspring performance increases the validity of using egg cortisol as a metric of broodstock or population health.

6.2 Environmental matching

From understanding the trajectory of diseases in humans as "predictive adaptive responses" (Gluckman and Hanson 2004) or analyzing the "anticipatory parental effects" in non-human animals and plants (Uller et al. 2013), there has been a recent surge in dialogue on the adaptive potential of maternal effects (i.e., responses of females induce offspring phenotypes deemed to be beneficial in anticipation of future circumstances). Dufty et al. (2002) postulated that the maternal endocrine system could mediate adaptive effects on offspring phenotype. Indeed, Meylan et al. (2012) focus on GCs as the candidate hormone for "hormonally mediated maternal effects" and Sheriff and Love (2013) identify GCs as "maternally-derived stress" or MDS signals. In fishes, Nesan and Vijayan (2013*a*) stated that the maternal deposition of cortisol and GR transcripts into fish eggs could serve as a "mechanism for transmitting [information] from stressed mothers to progeny." Increasingly, researchers have proposed that offspring exposure to MDS (*via* elevated pre-natal GCs) can induce adaptive phenotypic outcomes in offspring if the stressful environment inducing MDS in the mother is shared temporally or spatially by offspring (i.e., environmental matching; Love and Williams 2008). To test the adaptive potential of MDS requires an environmentally-relevant manipulation of MDS, that is, raising offspring in a "matched" stressful environment and then following fitness in the offspring (Sheriff and Love 2013). Selection for egg cortisol as a MDS signal will be favored in species where the maternal environment accurately predicts the environment of progeny in space or time

566 (Love et al. 2013). Unfortunately, to date the effects of elevated egg cortisol on offspring
567 phenotype in fish (Table 2) have been predominantly tested under neutral or benign conditions
568 (i.e., offspring are typically reared in unmanipulated common-garden environments). These
569 conditions may be "mismatched" to the information transmitted *via* the MDS signal in the egg
570 (i.e., elevated cortisol concentrations). There is great opportunity to enrich our understanding of
571 maternal effects by designing experiments that evaluate and interpret egg hormone-mediated
572 offspring phenotypes in light of the quality of the offspring's future environment (see Figure 1 in
573 Uller et al. 2013).

574

575 ***6.3 Adult phenotype and fitness***

576 The majority of studies to date have focused on relationships between egg cortisol
577 concentrations and offspring phenotype during early development *versus* the adult phenotype
578 (Table 2). There are both logistical (e.g., complexity/expense of rearing offspring to sexual
579 maturity) and phenological reasons for concentrating on effects of egg cortisol at early life
580 stages. Still, it is worthwhile to investigate how egg cortisol-mediated effects on early offspring
581 phenotype correlate with adult phenotype (and fitness). Phenotypic effects of experimentally-
582 elevated egg cortisol *via* maternal intraperitoneal injection were detected in farmed Atlantic
583 salmon 1.5 to 2 years after hatching (e.g., craniofacial and tissue abnormalities, reactivity to a
584 confinement stressor; Eriksen et al. 2011, 2013). Egg cortisol-mediated effects on offspring
585 phenotype early in life may set a developmental trajectory that dictates the adult phenotype. For
586 example, exogenously elevating egg cortisol can reduce offspring size in salmonids (Table 2),
587 which may be linked with changes to growth early in embryogenesis (Li et al. 2010), and

588 juvenile growth rates correlate with adult reproductive success (e.g., offspring survival; Burton et
589 al. 2013*b*). The adult phenotype can be an interaction between early life hormonal influences
590 (i.e., egg cortisol concentration/maternal signal) and the environment (see Figure 1 in Dufty et al.
591 2002). Alternatively, egg cortisol-mediated effects may restrict phenotypic flexibility, and the
592 offspring phenotype programmed early in life by egg cortisol titers may persist to adulthood.
593 Evaluating how egg cortisol affects the phenotype of adult offspring will fill a current knowledge
594 gap, but discerning the legacy effects of egg cortisol may be difficult due to inherent genetic
595 effects and accumulating environmental input. Given that little information is available regarding
596 the long-term effects of offspring exposure to elevated egg cortisol it is perhaps not surprising
597 that limited research has been conducted linking these effects to variation in adult fitness (e.g.,
598 survival to sexual maturation, age at reproduction, gamete quality).
599

600 **7. Conclusion**

601 Glucocorticoids in the eggs of oviparous animals, such as fishes, are not physiologically
602 static entities; levels vary within and across mothers, as well as across species. While the
603 environmental and ecological pressures driving variation in GC concentrations remain elusive,
604 this variation exerts diverse effects on biological functions of developing offspring, which has
605 the potential to interact with environmental variation to contextually impact offspring fitness. An
606 individual egg is but a fraction of a fish's mass, yet the contents of these globular morsels are the
607 "building blocks" progeny are given to develop, grow, and survive to independence. To fully
608 appreciate the phenotypic effects of egg GCs on the offspring of fishes, there are a number of
609 theoretical and experimental factors to encompass into future scientific inquiry. An integrative

610 approach to examining how egg GCs affect offspring phenotype can yield valuable insight into
611 hormonally-mediated intergenerational processes and population fitness.

612

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619

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Table 1. Egg cortisol concentrations of fishes. The common methodology utilized to quantify cortisol content of eggs is homogenization of pooled eggs, followed by extraction of the hormone using diethyl ether or ethyl acetate. Extracted samples are then analyzed with radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA). Number of females refers to the number of individuals that eggs were obtained from. Dashes (-) indicate data were not reported. Origin of the females was unknown (origin not reported), research broodstock (females held and bred in a laboratory facility), hatchery (females held and bred in a hatchery facility), commercial supplier (females obtained from commercial breeder), farmed (females obtained from fish farm), or wild-caught (females caught from natural water source). Time point refers to the time at which cortisol concentration was quantified (unfertilized [uf], at fertilization, or 1 hour post-fertilization (1 hpf)), or if cortisol was measured in ovarian tissue (ovarian). Mean ± SE and range (in brackets) of egg cortisol concentration (ng g^{-1}, see superscripts for exceptions and conversions) are presented where possible. If values were not presented in the text of published manuscripts average values were estimated from figures (indicated with a tilde mark; ~).

Species	Number of females; origin	Time point	Egg cortisol (ng g^{-1})	Reference
Brown trout (*Salmo trutta*)	3 farmed	uf	46.2 ± 13.1	Sloman 2010
Brown trout (*Salmo trutta*)	15 hatchery	uf	30.3 ± 9.3 (3.2-122.5)	Burton et al. 2011
Brook trout (*Salvelinus fontinalis*)	3 farmed	uf	~3.5[a]	Ghio et al. In press
Rainbow trout (*Oncorhynchus mykiss*)	5 research broodstock	uf	60 ± 8[b]	Li et al. 2010
Rainbow trout (*Oncorhynchus mykiss*)	10, 10 research broodstock	uf	23.1 ± 4.7[c] 16.7 ± 3.1[d]	Andersson et al. 2011
Rainbow trout (*Oncorhynchus mykiss*)	3 research broodstock	uf	5.1 ± 0.1	Ghaedi et al. 2013

Species	Number of females; origin	Time point	Egg cortisol (ng g^{-1})	Reference
Atlantic salmon (*Salmo salar*)	10, 15 farmed	uf	13 ± 87[e] 26 ± 46[ef] (21-558)	Eriksen et al. 2013
Chinook salmon (*Oncorhynchus tshawytscha*)	23 farmed	uf	20.6 ± 3.3 (2.4-57.1)	Capelle, unpublished data
Chinook salmon (*Oncorhynchus tshawytscha*)	12, 7 wild-caught	uf	12.1 ± 1.0[g] (6.3-17.2) 22.1 ± 8.9[h] (3.7-62.8)	Capelle, unpublished data
Coho salmon (*Oncorhynchus kisutch*)	7 hatchery	uf	9.9 ± 0.9	Stratholt et al. 1997
Coho salmon (*Oncorhynchus kisutch*)	15 hatchery	uf	9.4 ± 2.2 (1.1-30.5)	Sopinka et al. 2015*b*
Sockeye salmon (*Oncorhynchus nerka*)	17, 20 wild-caught	uf	8.7 ± 1.4 (2.7-32.8) 11.6 ± 1.7[i] (3.2–28.3)	Sopinka et al. 2014; 2016*a*
Persian sturgeon (*Acipenser persicus*)	1 wild-caught	uf	3.6 ± 0.7	Falahatkar et al. 2014
White sturgeon (*Acipenser transmontanus*)	2 farmed	uf	21.5 ± 3.5	Simontacchi et al. 2009
Atlantic cod (*Gadus morhua*)	9 research broodstock	uf[j]	~1[a]	Kleppe et al. 2013

Species	Number of females; origin	Time point	Egg cortisol (ng g^{-1})	Reference
Common carp (*Cyprinus carpio*)	1 unknown origin	uf	~208[k]	Stouthart et al. 1998
Silver carp (*Hypophthalmichthys molitrix*)	1 hatchery	uf	155.7 ± 0.5	Kausar et al. 2013
Threespined stickleback (*Gasterosteus aculeatus*)	7 wild-caught	uf	12.6 ± 1.6	Paitz et al. 2015
Damselfish (*Pomacentrus amboinensis*)	150 wild-caught	ovarian	(0.3-76.0)	McCormick 1998
Fathead minnow (*Pimephales promelas*)	23 research broodstock	ovarian	190	DeQuattro et al. 2012
Great sturgeon (*Huso huso*)	5 farmed	ovarian[l]	13.5 ± 3.9	Poursaeid et al. 2012
Largemouth bass (*Micropterus salmoides*)	19 wild-caught	ovarian	11.1 ± 0.8	O'Connor et al. 2013
Asian seabass (*Lates calcarifer*)	~25 farmed	at fertilization	2.20[m] 1.20[n] 0.62[o]	Sampath-Kumar et al. 1995
Chum salmon (*Oncorhynchus keta*)	1 research broodstock	at fertilization	20	de Jesus and Hirano 1992
Japanese flounder (*Paralichthys oliwceus*)	- research broodstock	at fertilization	2.5	de Jesus et al. 1991

Species	Number of females; origin	Time point	Egg cortisol (ng g^{-1})	Reference
Rainbow trout (*Oncorhynchus mykiss*)	6, 6 hatchery	at fertilization	1.4; 6.0	Barry et al. 1995
Yellow perch (*Perca flavescens*)	6 research broodstock	at fertilization	~1[p]	Jentoft et al. 2002
Zebrafish (*Danio rerio*)	- commercial supplier	at fertilization	~600[q]	Nesan and Vijayan 2012
Red drum (*Sciaenops ocellatus*)	- research broodstock	1 hpf	0.2 ± 0.2	Applebaum et al. 2010

[a]ng mL^{-1}. [b]converted from ng egg^{-1} to ng g^{-1} based on egg size of 0.05 g, estimated from Blom and Dabrowski (1995). [c]concentration in eggs collected from females selected for high stressor-induced plasma cortisol. [d]concentration in eggs collected from females selected for low stressor-induced plasma cortisol. [e]converted from ng egg^{-1} to ng g^{-1} based on egg size of 0.10 g from Berg et al. (2001). [f]females were injected with 1 mg kg^{-1} coconut oil (sham). [g]females migrate ~650 km from the ocean to reach freshwater spawning grounds. [h]females migrate ~800 km from the ocean to reach freshwater spawning grounds. [i]females were wild-caught and held in captivity for ~6 weeks. [j]females had a sham osmotic surgically-inserted 3 weeks prior to egg sampling. [k]converted from pg 20^{-1} eggs to ng g^{-1} based on egg size of 1.2 mg from Stouthart et al. (1998). [l]females were injected every 6 weeks for 6 months with two gelatinous intraperitoneal capsules containing 0.2 mL cocoa butter (sham), follicles within in sampled ovaries were pre-vitellogenic. [m]average concentration in eggs collected in July and October 1991, and March 1992. [n]concentration in eggs collected January 1992. [o]concentration in eggs collected in February 1992. [p]1-cell stage embryos, converted from pg $embryo^{-1}$ to ng g^{-1} based on egg size of 0.006 mg from Markovich et al. (2007). [q]converted from pg $embryo^{-1}$ to ng g^{-1} based on egg size of 0.04 g, from Brown et al. (2009).

Table 2. Effects of direct (egg: microinjection, bath) and indirect (female: intraperitoneal injection, osmotic pump, soaked food pellet) manipulation of egg cortisol levels. Eggs were bathed at fertilization with cortisol-dosed water or prior to fertilization by dosing the ovarian fluid. Phenotypic traits described are traits observed in offspring reared from cortisol-treated eggs/females. Mean (± SE) concentration of cortisol is reported for 1) untreated and treated eggs between 0 and 2 hours post-fertilization (hpf) when water used to activate sperm was dosed with cortisol, 2) unfertilized eggs after 3 hours of treatment in ovarian fluid/salt solution dosed with and without cortisol, and 3) ovarian tissue/unfertilized eggs sampled from females with intraperitoneal implants, osmotic pumps, or fed food pellets soaked with cortisol (1-21 days post-treatment). If values were not presented in the text of published manuscripts, average values were estimated from figures (indicated with a tilde mark; ~). Survival effects are reported for embryos (survival up to first feeding/yolk sac absorption). Body size effects (body length and/or body mass) are reported for alevin (yolk sac present) and fry (full yolk-sac absorption at time of first feeding) life stages or according to days post-fertilization (dfp) or months post-hatch (mph). Dashes indicate the parameter was not reported.

Species	Manipulation (dose; duration)	Untreated egg concentration (ng g^{-1})	Treated egg concentration (ng g^{-1})	Embryonic survival effects	Body size effects	Other phenotypic effects	Reference
Brown trout (*Salmo trutta*)	bath 0 or 470 ± 185 ng mL^{-1}; ovarian fluid, 3 hours	46.2 ± 13.1	699.0 ± 46.4	no effect	no effect (28, 43, 58, 87, 292 dpf)	elevated oxygen consumption and ammonia excretion rate, more aggressive displays, and different response to a maze	Sloman 2010
Brown trout (*Salmo trutta*)	bath 0 or 200 ng mL^{-1}; at fertilization, 2 hours	11.1 ± 0.7	55.0 ± 5.4	-	no effect (alevin), smaller (fry)	reduced aggression and lower social status score; no effect on standard metabolic rate, territory quality or competitive ability	Burton et al. 2011
Brook trout (*Salvelinus fontinalis*)	bath 500 ng mL^{-1}; salt solution, 3 hours	~3.5[a]	~200[a]	-	-	no effect on boldness, spatial learning or memory, or neophobia	Ghio et al. In press

Species	Manipulation (dose; duration)	Untreated egg concentration (ng g^{-1})	Treated egg concentration (ng g^{-1})	Embryonic survival effects	Body size effects	Other phenotypic effects	Reference
Rainbow trout (*Oncorhynchus mykiss*)	bath 0, 200 or 1000 ng mL^{-1}; at fertilization, 1 hour	~4	~7; ~7	-	-	attenuated plasma cortisol response	Auperin and Geslin 2008
Rainbow trout (*Oncorhynchus mykiss*)	bath 0, 200 or 1000 ng mL^{-1}; ovarian fluid, 3 hours	64±6[b]	94±6[b] 144±9[b]	no effect (low dose), reduced (high dose)	no effect (fry), larger (low dose, 102, 117 dpf), no effect (high dose, 102, 117 dpf)	ontogenetic variation in lysozyme activity, intelectin levels, and transcript abundance of genes for growth and immunity	Li et al. 2010, 2011, 2012*b*
Rainbow trout (*Oncorhynchus mykiss*)	bath 0 or 20000 ng mL^{-1}; at fertilization, 55 min	74 ± 13[b]	725 ± 148[b]	no effect	no effect (146 dpf)	ontogenetic variation in startle response; lower basal plasma cortisol	Colson et al. 2015
Atlantic salmon (*Salmo salar*)	injection 0, 50 or 100 mg kg^{-1}; 6 days	37.6 ± 28.7[a]	123.1 ± 26.9[a] 123.7 ± 25.4[a]	no effect	smaller (alevin, fry), larger (high dose, 4 mph, 8.5 mph), no effect (10 mph)	increased fluctuating asymmetry, less active in novel environment	Eriksen et al. 2006, 2007, 2008; Espmark 2008

Species	Manipulation (dose; duration)	Untreated egg concentration (ng g^{-1})	Treated egg concentration (ng g^{-1})	Embryonic survival effects	Body size effects	Other phenotypic effects	Reference
Atlantic salmon (*Salmo salar*)	injection 0 or 100 mg kg^{-1}; 7 days	26 ± 46[c]	681 ± 55[c]	-	no effect (11 mph)	variation in response to confinement (more active 4 mph, less active 18 mph), increased occurrence of morphological malformations, no effect on feeding or social status	Eriksen et al. 2011, 2013
Chum salmon (*Oncorhynchus keta*)	bath 0 or 1000 ng mL^{-1}; at fertilization, 2 hours	5.5 ± 0.5	22.3 ± 1.4	no effect	no effect (178 dpf)	no effect on swimming duration	Sopinka et al. 2016*b*
Coho salmon (*Oncorhynchus kistuch*)	bath 0 or 600 ng mL^{-1}; at fertilization, 2 hours	37.0 ± 5.4	232.7 ± 13.9	no effect	no effect (alevin)	-	Stratholt et al. 1997
Coho salmon (*Oncorhynchus kistuch*)	bath 0 or 1000 ng mL^{-1}; at fertilization, 2 hours	9.3 ± 2.2	33.0 ± 1.0	no effect	-	higher dominance and boldness scores	Sopinka et al. 2015*b*
Sockeye salmon (*Oncorhynchus nerka*)	0 or 1000 ng mL^{-1}; at fertilization, 2 hours	10.8 ± 4.4	33.7 ± 1.4	no effect	smaller (182 dpf)	no effect on swimming duration	Sopinka et al. 2016*b*
Atlantic cod (*Gadus morhua*)	osmotic pump 0 or 30 mg; 3 weeks	~1[b]	~20[b]	no effect	-	variation in expression of genes for cytogenesis	Kleppe et al. 2013

Species	Manipulation (dose; duration)	Untreated egg concentration (ng g^{-1})	Treated egg concentration (ng g^{-1})	Embryonic survival effects	Body size effects	Other phenotypic effects	Reference
Damselfish (*Pomacentrus amboinensis*)	injection 0, 50 or 100 μg g^{-1}; 5-7 days	~2[d]	~6 [d]; ~8[d]	-	smaller (larval)	-	McCormick 1998
Zebrafish (*Danio rerio*)	microinjection 0 or 32000 ng mL^{-1}; at one-cell stage	-	-	no effect	-	reduced transcript abundance of genes for cardiac development, and resting and post-stressor exposure heart rate	Nesan and Vijayan 2012
Zebrafish (*Danio rerio*)	microinjection 0 or 32000 ng mL^{-1}; at one-cell stage	-	-	no effect	larger (48, 72 hpf), no effect on head-trunk angle	higher whole body cortisol content, absence of stress-induced changes in whole body cortisol, increased and reduced transcript abundance of stress axis genes, no effect on transcript abundance of corticosteroid receptor genes	Nesan and Vijayan 2016
Zebrafish (*Danio rerio*)	soaked food pellet 25 μg g^{-1} body mass; 5 days	23.1 ± 6.3[d] 0.0005-0.01[e]	48.0 ± 6.6[d] 0.001-0.04[e]	-	-	-	Faught et al. 2016

[a]ng mL^{-1}. [b]converted from ng egg^{-1} to ng g^{-1} based on egg size of 0.05 g, estimated from Blom and Dabrowski (1995). [c]converted from ng egg^{-1} to ng g^{-1} based on egg size of 0.1 g, estimated from Berg et al. (2001). [d]ovarian tissue. [e]range of concentrations (ng $embryo^{-1}$) measured in embryos (1 hpf) collected 1 to 10 days following the 5 day feeding of cortisol-soaked food pellets.

Figure 1. Schematic of effects of maternal stressor exposure and egg cortisol on offspring phenotype. (A) Maternal stressor exposure activates the hypothalamic-pituitary-interrenal (HPI) axis. The hypothalamus releases corticotropin-releasing factor (CRF) which stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary. ACTH binds to receptors on the interrenal cells in the head kidney initiating a biochemical cascade resulting in the synthesis of cortisol. Circulating cortisol binds to glucocorticoid receptors (GRs) on target tissues and also reaches developing follicles in the female's ovaries. (B) Within the fertilized egg, maternally-derived cortisol is thought to bind to GRs translated from maternally-derived GR transcripts. Once bound, the ligand-activated GR is hypothesized to induce changes in abundance of embryonic transcripts associated with developmental pathways, resulting in altered offspring phenotype (Section 3.1). (C-I) Stressor-induced increases in circulating maternal cortisol can be associated with increased concentrations of ovarian/egg cortisol (Section 5.3). Maternal transcripts, including transcripts for GRs, also enter the vitellogenic follicle. (C-II) There is evidence for metabolism of cortisol to cortisone in the thecal/granulosa layer of follicles by 11β-hydroxysteroid dehydrogenase 2 (11*βHSD2)*, as well, metabolism of cortisol to cortisol- and cortisone-sulphates by glucocorticoid sulphotransferase (GST) (Section 3.2). (C-III) Efflux of cortisol via transmembrane ATP-binding cassette (ABC) transporters is also observed in newly fertilized eggs (Section 3.2).

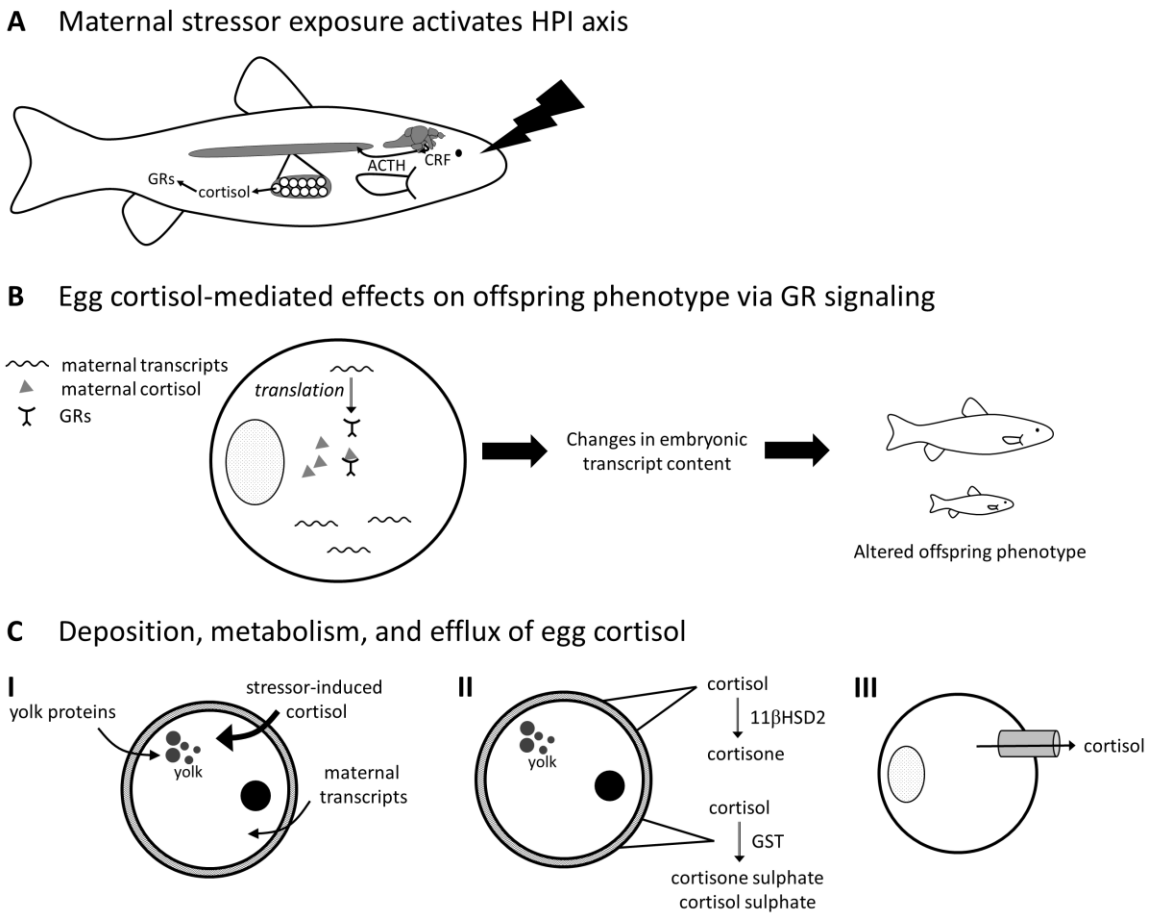


Figure 2. Schematic of oogenesis. The previtellogenic follicle (I) is surrounded by a follicular layer comprised of thecal and granulosa cells, and contains the germinal vesicle (●). During vitellogenesis (II), yolk proteins (e.g., vitellogenins) are incorporated into the developing follicle and processed into yolk. Cortisol, a lipophilic steroid, enters the vitellogenic follicle and accumulates in the yolk via diffusion or co-entry with yolk proteins. The movement of cortisol between the follicle and maternal circulation is thought to be bidirectional. Lipids, vitamins, and maternal transcripts are also incorporated into the follicle during vitellogenesis. Due to the incorporation of lipids and proteins during vitellogenesis, the follicle undergoes significant growth. Hydration of the follicle (III) occurs post-vitellogenesis whereby water and ions are taken up into the mature follicle and the germinal vesicle breaks down. Yolk proteins are also hydrolyzed into free amino acids (a.a.). Following hydration, ovulation occurs (IV) whereby the mature oocyte, containing an oil droplet (◍) , is released from the follicle into the abdominal cavity. The ovulated oocyte contains nutritional, molecular, and hormonal (e.g., cortisol) components necessary for proper embryo development. Figure adapted from Cedrea et al. (2008).

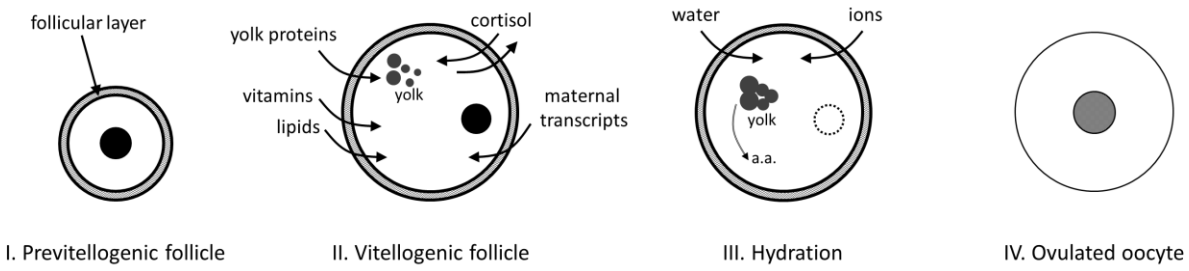


Figure 3. Log_{10} cortisol concentration in unfertilized eggs and log_{10} maternal body condition of Pacific salmon; hatchery coho salmon *Oncorhynchus kisutch*, open circles (Pearson correlation: r^2=0.30, n=13, P=0.05); farmed Chinook salmon *O. tshawytscha*, filled circles (r^2=0.10, n=27, P=0.10); wild-caught sockeye salmon *O. nerka*, squares (r^2=0.26, n=48, P=0.0002, line shown). Egg cortisol was quantified following Sopinka et al. (2014; 2015*b*). Maternal body condition was calculated following Fulton's condition factor, K: [body mass (g) fork length^3 (cm)]$^{-1}$ x 100%. See Section 6.1 for further details.

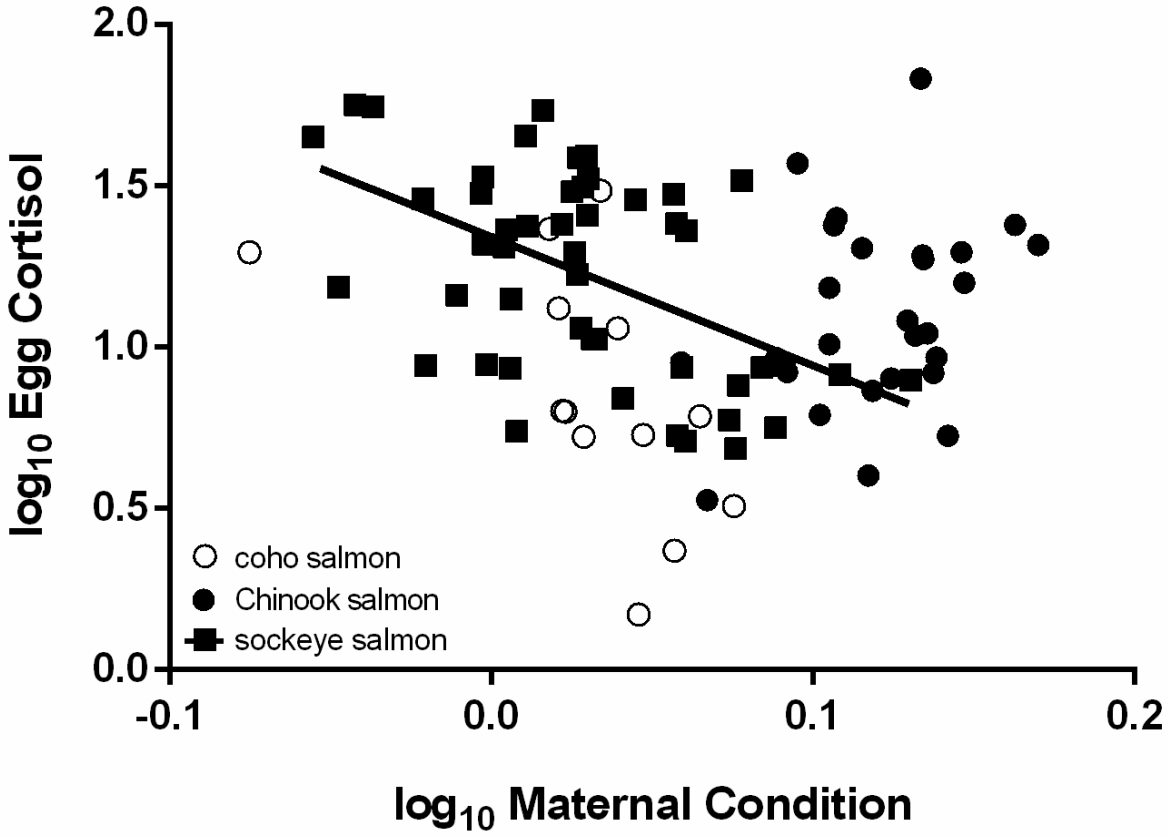


Figure 4. Proposed relationships between concentration of egg cortisol and offspring phenotypic performance. Offspring performance is optimal within an intermediate range of egg cortisol concentrations, and sub-optimal at both lower and higher concentrations of egg cortisol (A). Offspring performance is optimal at lower concentrations of egg cortisol and decreases linearly as egg cortisol concentrations increase (B). Offspring performance is maintained throughout a range of egg cortisol concentrations but decreases when concentrations increase above a specific threshold concentration (C). See Section 6.1 for further details.

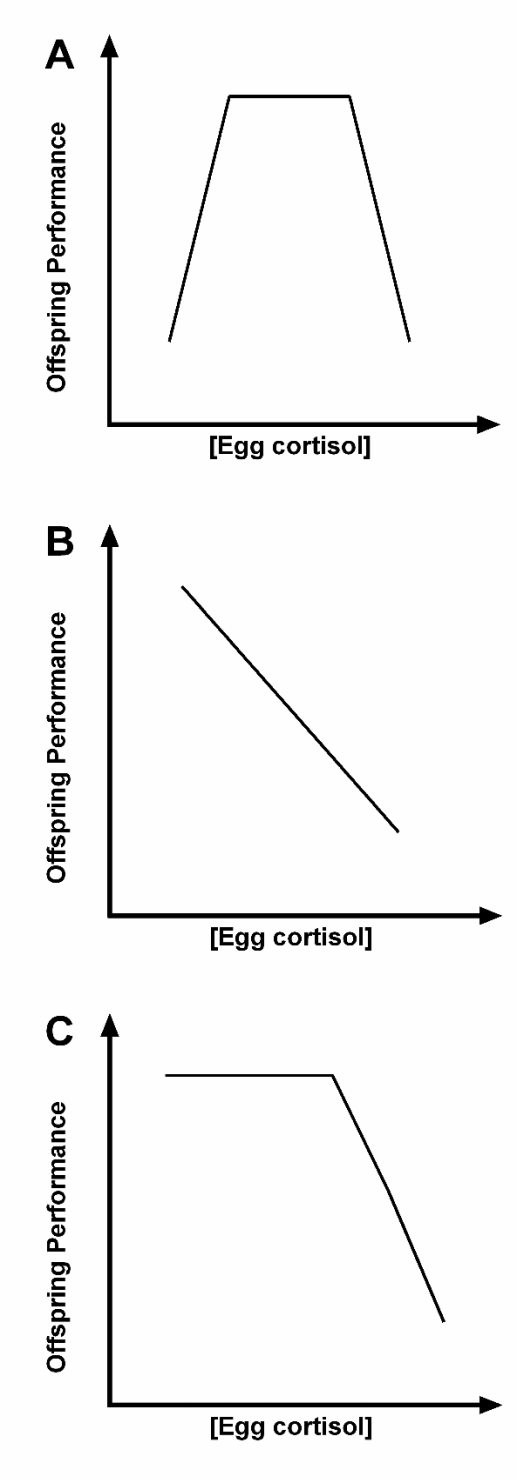


Figure 1

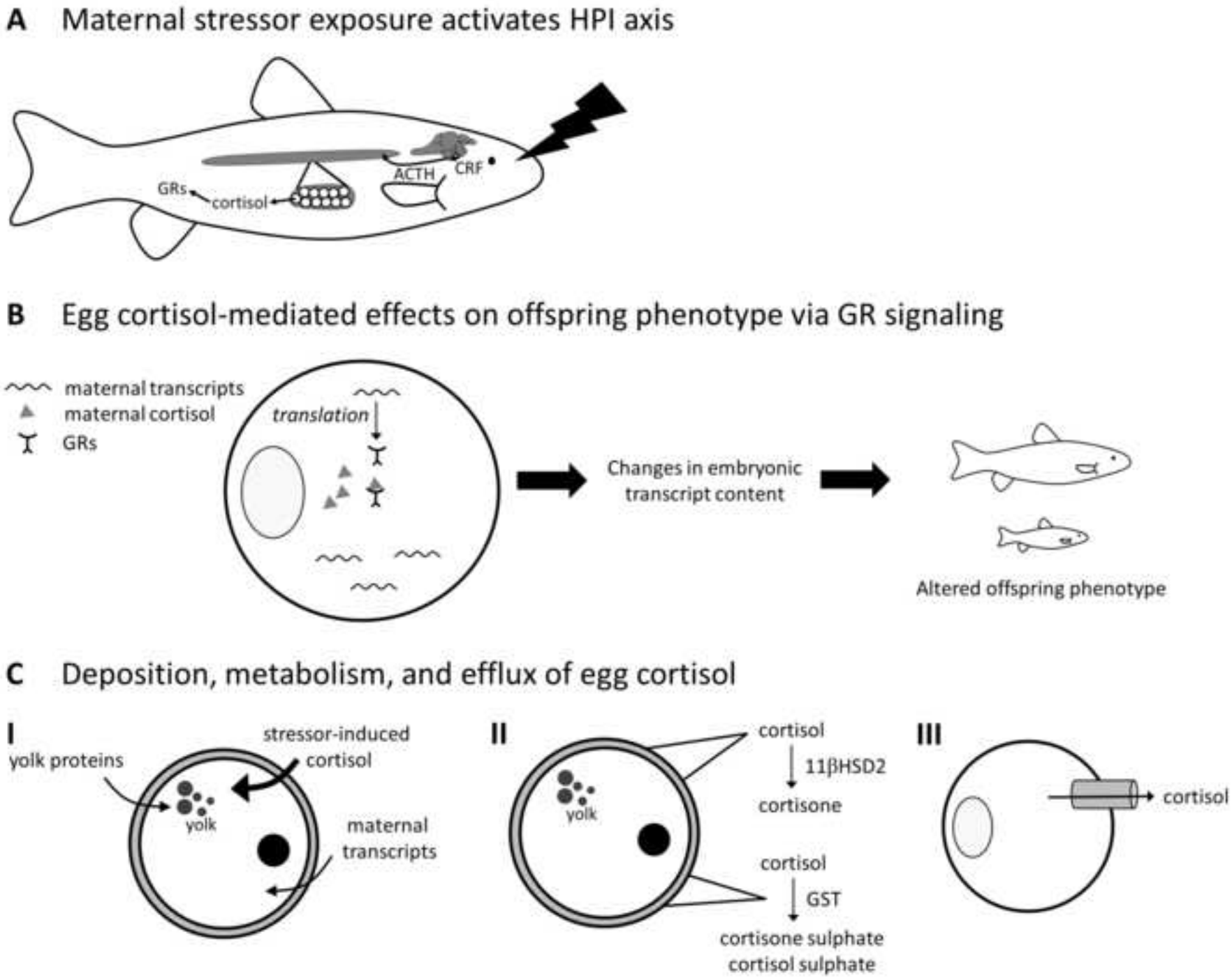

A Maternal stressor exposure activates HPI axis
GRs
cortisol
ACTH
CRF
B Egg cortisol-mediated effects on offspring phenotype via GR signaling
maternal transcripts
maternal cortisol
GRs
translation
Changes in embryonic transcript content
Altered offspring phenotype
C Deposition, metabolism, and efflux of egg cortisol
I
yolk proteins
stressor-induced cortisol
yolk
maternal transcripts
II
cortisol
11βHSD2
cortisone
yolk
cortisol
GST
cortisone sulphate
cortisol sulphate
III
cortisol

Figure 2

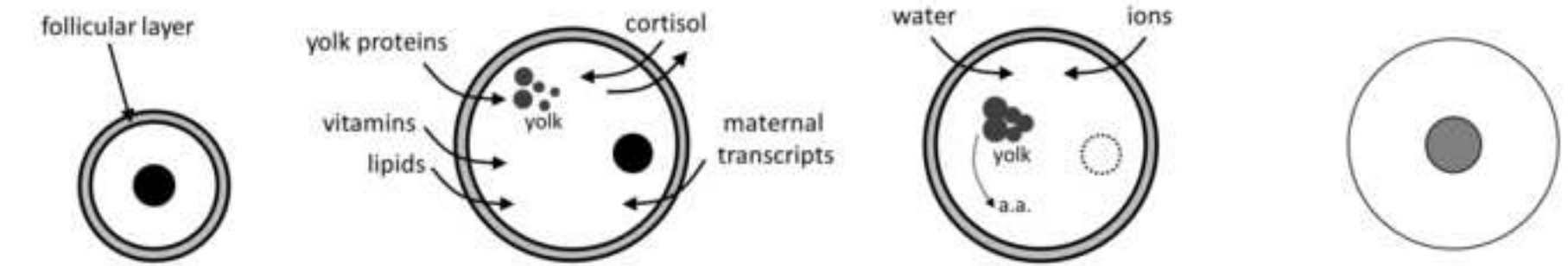

follicular layer
yolk proteins
cortisol
vitamins
yolk
lipids
maternal
transcripts
water
ions
yolk
a.a.
I. Previtellogenic follicle
II. Vitellogenic follicle
III. Hydration
IV. Ovulated oocyte

Figure 3

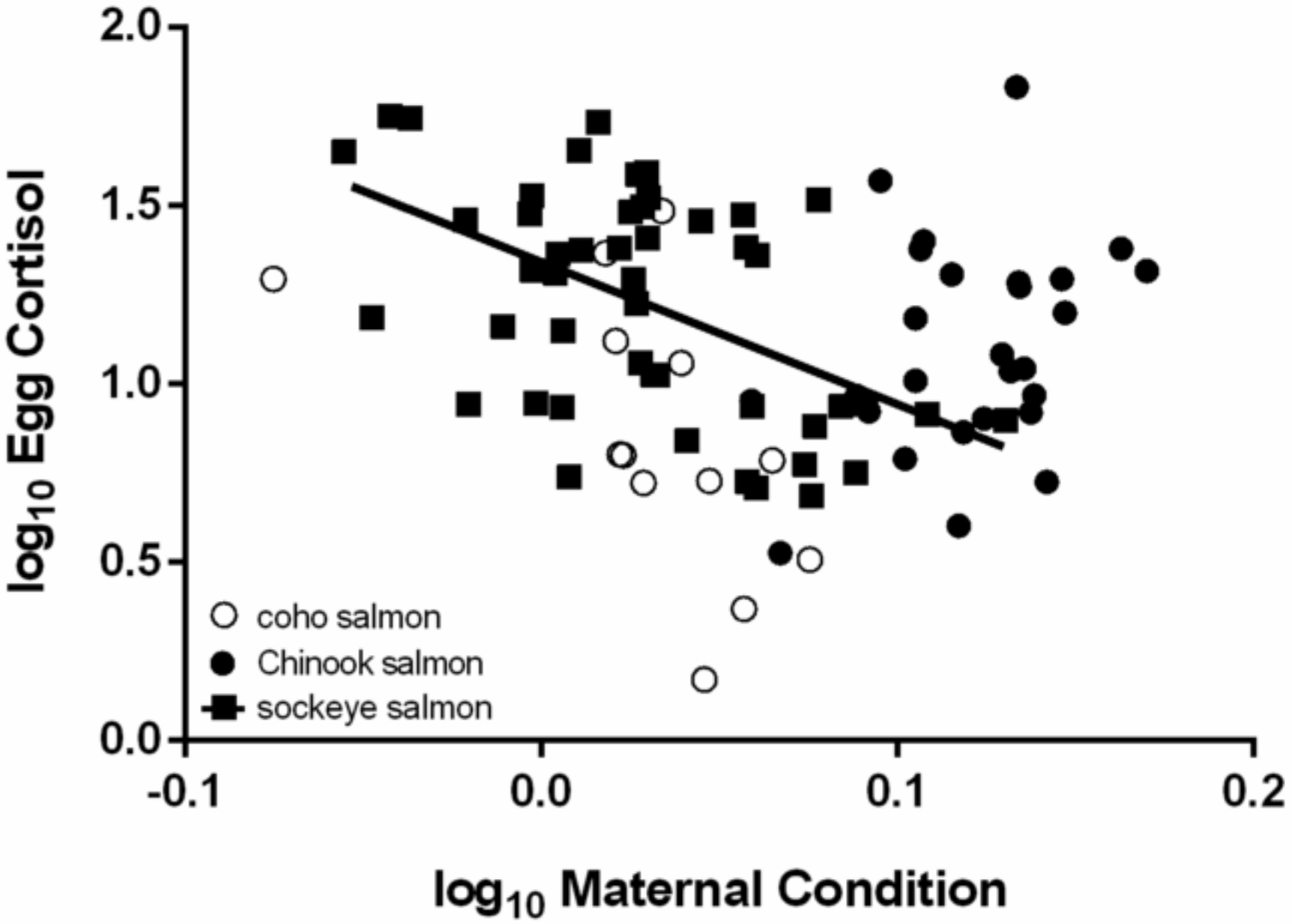
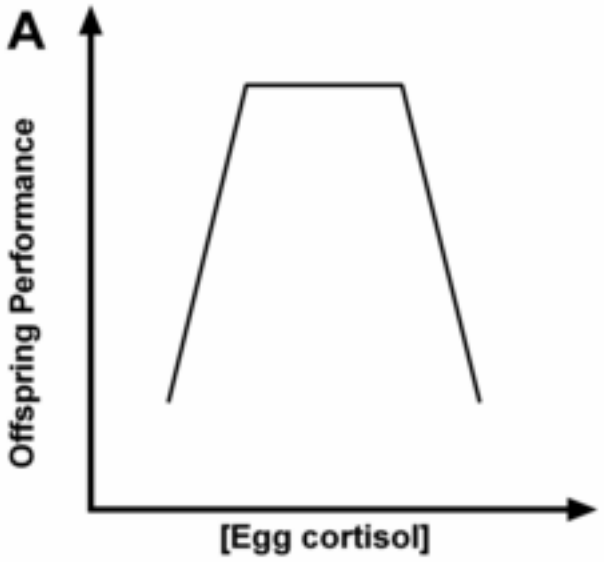
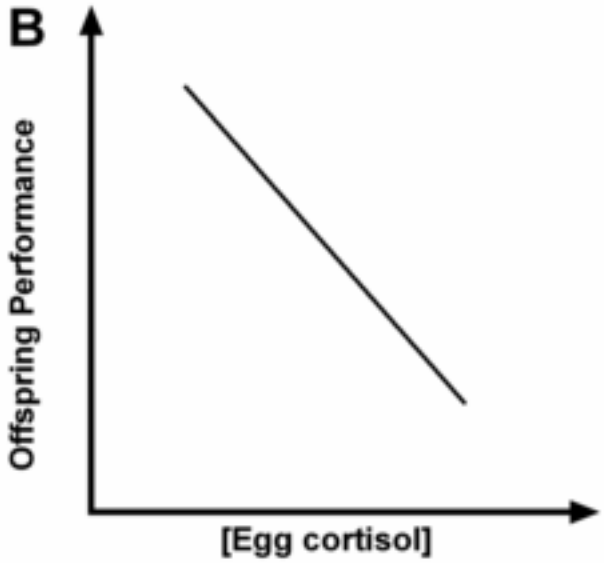
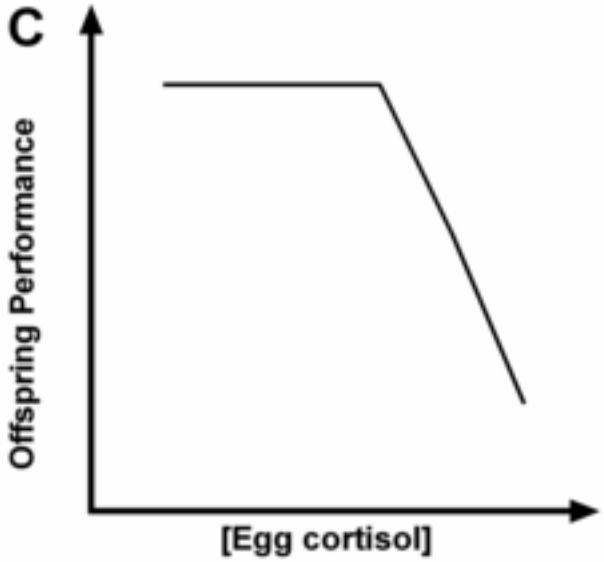

2.0
1.5
1.0
0.5
0.0
log10 Egg Cortisol
coho salmon
Chinook salmon
sockeye salmon
-0.1
0.0
0.1
0.2
log10 Maternal Condition

Figure 4


A
Offspring Performance
[Egg cortisol]


B
Offspring Performance
[Egg cortisol]


C
Offspring Performance
[Egg cortisol]

PBZ-16027R2

Responses to Reviewers

We have addressed the comments and outline how each issue was dealt with in the attached letter entitled "Response to the Reviewers". We have extensively reorganized the review taking into consideration the helpful comments of the Editorial Board Member and Reviewer 2. The current structure of the review incorporates additional and revised titles for different sections. We have moved sections from the Conclusion section to the main text of the review and streamlined text where possible. We hope this revised structure adequately improves the flow of the paper.

1. Introduction – brief introduction of stress which now focuses on fishes and also ends with a paragraph specifically describing the major topics that will be discussed in the review.
2. Maternal GCs and oviparity: what do we know from birds? – single paragraph summary of research in avian systems that can guide study in fishes.
3. Egg GCs in fishes – subdivided into two sections that have been moved from the Conclusion section; the first section (3.1) describes mechanisms of action of egg cortisol and the second section (3.2) describes metabolism of egg cortisol.
4. Natural variation in egg cortisol – subdivided into two sections; the first section (4.1) describes the natural variation in egg cortisol among fishes and outlines potential drivers of this variation; the second section (4.2) describes effects of natural variation in egg cortisol on offspring phenotype and fitness.
5. Experimental manipulation of egg cortisol – subdivided into three sections; the first section (5.1) describes methodologies used to manipulate egg cortisol; the second section (5.2) describes effects of experimentally-manipulated egg cortisol on offspring phenotype and fitness; the third section (5.3) describes studies that stressor-exposed mothers and assessed egg cortisol and phenotypic effects on offspring.
6. Future research directions – subdivided into three sections; the first section (6.1) describes future research related to quantifying natural variation in egg cortisol; the second section (6.2) describes future research related to environmental matching; the third section (6.3) describes future research related to assessing adult offspring phenotype
7. Conclusion – single paragraph conclusion of review

We look forward to hearing from you regarding the current version of the manuscript.

RESPONSE TO THE REVIEWERS

Editorial Board Member Comments

I agree with the reviewer that the review paper could still benefit from a bit more organization to address the reviewer's concerns and help with flow, but I think this is more about reorganization and fine-tuning and so may not be as onerous as it first sounds.

I think in general, the review paper could benefit from more headings that help to streamline the topics being covered The first paragraph nicely introduces the topic and line 70 indicates what the review is about. Immediately following this, please add a paragraph that describes the major topics that will be discussed in the remainder of the review (a, b, c and d concluding with a section of future directions) to provide the reader with a more specific description and outline of what is to come.

Response: At the end of the Introduction (Section 1), we now include a paragraph that describes the major topics/Sections that will be discussed in the remainder of the review, with specific reference to the Section numbers (see lines 98-107).

Thus, rather than have 5 pages of introduction that covers a lot of territory under one heading, have 2 or 3 headings in that section. For example, the pages that follow from line 70 jump into information on birds and mammals, which is an area we know more about and is important for laying basic principles. If that section had its own title to indicate this, and in an early paragraph following line 70 it had been indicated how important this information is it would help to make it clear why it is being discussed in a review on fish. Adding more headings in general throughout, will help to organize the material better to address the concerns of reviewer 1.

Response: We now include additional headings in the introductory paragraphs to help guide readers and provide the reader with a more specific description of the content to follow. Specifically, we mention why bird literature is being briefly reviewed at the end of the Introduction (see lines 99-101). We also include the section heading "1.1 Maternal GCs and oviparity: what do we know from birds?", providing the reader with the relevance of this section on birds to the broader scope of this review on fishes. We also indicate at the end of this paragraph (now truncated) that "In turn, this growing body of literature in avian systems has supported and enhanced study of the effects of maternal GCs on offspring in other oviparous taxa, including fishes." (see lines 109-132).

I also agree that the conclusions section is far too long and much of that information is presented for the first time in that section and should be reviewed in the main body of the text under appropriate headings and then based upon that information, the future directions for that topic in the conclusions section will be more effective.

Response: We have moved information from the Conclusions section on the mechanism of action of egg GCs and metabolism of egg GCs to the main text of the manuscript (now Sections 3.1 and 3.2, respectively).

Reviewer 2 Comments

General comments:
Overall, this review has been greatly improved, and acceptable, but it is still poorly organized.

- The best review of literature is section 5, yet for some reason it is labelled as "conclusions and future directions". I suggest moving almost the entirety of this section to the main part of the review, and the beginning part of the review streamlined so that there are not three separate sections on phenotype.

Response: We have moved information from the Conclusions section on the mechanism of action of egg GCs and metabolism of egg GCs to the main text of the manuscript (now Sections 3.1 and 3.2, respectively). We have streamlined the content on phenotype by removing how we defined phenotype and limiting discussion of adult phenotype as a future research direction (Section 6.3).

- The diagrams are an excellent addition to this manuscript, but little explanation is provided in the text - so, please expand.

Response: We have expanded the explanation of the diagrams in text and now reference the figures as follows:

Fig. 1*a* at lines 55-58, 74-76, 145-149.
Fig. 1*b* at lines, 77-78, in Section 3.1 (see lines 172-173, 185-187, 191-192).
Fig. 1*c* at lines 77-78, 157-159, in Section 3.2 (see lines 231-232, 261-264)
Fig. 2 in Section 3 (see lines 133-142).

- Despite the title, this review is heavily biased on the detrimental effects of "excess" cortisol and does not address until the very end the necessity of cortisol. A few sentences to clarify this upfront would help. (like section 5.2 - consider moving/incorporating this section). Also, specifics of GR signaling should already be mentioned in the introduction, rather than wait until section 4.

Response: We have added a sentence regarding the necessary of cortisol in eggs for proper offspring development earlier in the review (see lines 135-136). We have now moved sections on GR signaling and metabolism of egg GCs (now Sections 3.1 and 3.2, respectively) earlier in the review.

- The introduction can be streamlined; it is unfocused. While I understand the reason for the

focus on birds, the extent of discussion is distracting for a fish-specific review (for instance, Lines 91-112 is unnecessary as this is not a review on birds. If it is comparative in scope then you should mention that in the title and expand the mammalian studies as well. Otherwise it is very distracting!). My suggestion would be reduce the part on birds and just focus on fish. The introduction should set the stage for the rest of the manuscript and that is not the case with all the distractions.

Response: We have removed content in this section of the Introduction, and with the addition of a title for this section (1.1 Maternal GCs and oviparity: what do we know from birds?) have provided the reader with the relevance of this section on birds to the broader scope of this review on fishes. We also indicate at the end of this paragraph (now truncated) that "In turn, this growing body of literature in avian systems has supported and enhanced study of the effects of maternal GCs on offspring in other oviparous taxa, including fishes." (see lines 109-132).

Specific comments:

- Ln 77 - citation required for the following statement "…GCs are furthermore implicated in cross-generational regulation of phenotypic traits"

Response: We have now removed this sentence in efforts to streamline this section of the Introduction.

- Ln 52 - the HPI/HPA axis is not the only coping mechanism. What about the adrenergic response and the rapid increase in catecholamines to deal with stressors?

Response: We have changed this sentence to read "One of the coping mechanisms encompassed within the vertebrate stress response is the activation of the HPA/-I axis (i.e., hypothalamic–pituitary–adrenal [HPA] axis in mammals, birds, reptiles (see Fig. 1 in Boonstra 2013); HP-interrenal [HPI] axis in fishes, Wendelaar Bonga 1997, [Fig. 1*a*]), resulting in the production of glucocorticoids (GCs)." (see lines 55-58).

- Ln 154 - 156 - you do not need to posit this, as it has already been shown in fish. You should directly be stating what was found in fishes - as in ln 60. Again, this is a review on fish and whether or not it was done first in birds is unnecessary and distracting!

Response: We have removed this sentence.

- Inter-generational and cross-generational - are these synonyms? If not, this is not clear.

Response: We have changed the wording to intergenerational.

Section 4

- Ln 386 - why is maternal and embryonic metabolism of cortisol in future directions as you are referring to it here. By this point you should have already discussed it.

Response: We have moved the section on metabolism of cortisol earlier to be read earlier in the review (now Section 3.2).

Section 5:
- Much of this section should be incorporated into the main text. Conclusion and future directions should be a short paragraph - not half the manuscript! There is much in this section that needs to be included earlier. I should not have to constantly refer to a section in the "conclusions" section when reading earlier sections to get a complete picture.
Response: We have moved the sections on mechanisms of action of egg GCs as well as metabolism of egg GCs (now Sections 3.1 and 3.2, respectively) to be read earlier in the review.

Minor comments:
- Rephrase sentence 59-61.
Response: We have amended this sentence, see lines 58-61.

- Line 73 "(" missing.
Response: We have added this bracket.

- Line 84-86. You may want to tone down the "well documented"! "Mechanisms" are not well documented.
Response: We have removed this sentence.

- Line 303-306. Presumptuous statement and indefensible, so rephrase it. Also, the use of the term "aquatic scientists" is odd, as opposed to what "terrestrial scientists", "bird scientists"? Please delete or rephrase it.
Response: We have rephrased sentences in this section (see lines 386-408).

- Line 313-314 - heated butter! Wouldn't that be a heat stress for fish residing in 10oC water temperature? The sentence doesn't make sense unless you substantiate clearly what you mean. This and the next few sentences just makes statements without putting into context what that means. For instance what is the advantage of the osmotic pumps. There are reasons for using various techniques, so don't just gloss over it. If that is the case might as well have a single sentence saying that several techniques, including blah blah blah, are used for manipulating cortisol levels in vivo, instead of highlighting a few.
Response: We have amended this section for clarification. We have guided readers to review papers that discuss the advantages and disadvantages of different methodologies (see lines 406-408).

- Lines 442-448. Not sure the connection between the two sentences. Yes they may be hyporesponsive to HPI activation, but that doesn't mean that cortisol wont bind to GR during that period! HPI hypoactivity, if I understand correctly, is meant to indicate a lack of cortisol

response to HPI activation.

Response: We have amended these sentences for clarification to "Maternal transcripts for GRs are detected in newly fertilized zebrafish (*Danio rerio*) eggs and extensive work on this model species has revealed the mechanistic actions of maternally-derived cortisol and GR transcripts in mediating offspring development (e.g., regulating development of the stress axis; Nesan and Vijayan 2013*a*,*b*). Pikulkaew et al. (2011) postulated that, in zebrafish, binding of maternally-derived cortisol to GRs, translated from maternal GR transcripts, was possible shortly after fertilization (Fig. 1*b*)." (see lines 168-173).