CORRECTION

Correction: Programming of the hypothalamic-pituitary-interrenal axis by maternal social status in zebrafish (Danio rerio)

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There were two errors published in J. Exp. Biol. 219, pp. 1734-1743.

In Table 1, the primers listed for the bottom two genes, β-actin and ubiquitin, are identical. The ubiquitin primers are incorrect – they should be:

F: gag cct tct ctc cgt cag tta g
R: cgc agg ttg ttg gtg tgt c

The accession number listed in Table 1 for ubiquitin is correct but the reference provided, Alsop and Vijayan (2008), is incorrect and should be Sun et al. (2010).


The authors apologise to the readers for any inconvenience this may have caused.
RESEARCH ARTICLE

Programming of the hypothalamic-pituitary–interrenal axis by maternal social status in zebrafish (Danio rerio)

Jennifer D. Jeffrey* and Kathleen M. Gilmour

ABSTRACT

The present study examined the effects of maternal social status, with subordinate status being a chronic stressor, on development and activity of the stress axis in zebrafish embryos and larvae. Female zebrafish were confined in pairs for 48 h to establish dominant/subordinate hierarchies; their offspring were reared to 144 h post-fertilization (hpf) and sampled at five time points over development. No differences were detected in maternal cortisol contribution, which is thought to be an important programmer of offspring phenotype. However, once zebrafish offspring began to synthesize cortisol de novo (48 hpf), larvae of dominant females exhibited significantly lower baseline cortisol levels than offspring of subordinate females. These lower cortisol levels may reflect reduced hypothalamic-pituitary–interrenal (HPI) axis activity, because corticotropin-releasing factor (crf) and cytochrome p450 side chain cleavage enzyme (p450sc) mRNA levels also were lower in larvae from dominant females. Moreover, baseline mRNA levels of HPI axis genes continued to be affected by maternal social status beyond 48 hpf. At 144 hpf, stress-induced cortisol levels were significantly lower in offspring of subordinate females. These results suggest programming of stress axis function in zebrafish offspring by maternal social status, emphasizing the importance of maternal environment and experience on offspring stress axis activity.

KEY WORDS: Dominance hierarchy, HPI axis, Fish, Offspring, Cortisol, Stress

INTRODUCTION

Competition over resources that are limited in an environment, such as food, advantageous territory and access to mates, drives the formation of social hierarchies in a wide range of animal taxa, including teleost fish. The formation of social hierarchies has been well studied across a range of teleost species (Johnsson et al., 2006). Among juvenile teleost fish, such as juvenile salmonids, competition for food-rich territory, and thus monopolization of food resources, is high. Among adult teleosts, competition for reproductive opportunities and reproductive territories becomes more significant. The development of social hierarchies tends to lower aggression levels within groups and these relationships generally remain linear and stable, although reversals are possible (Abbott and Dill, 1985; Johnsson, 1997).

The social status acquired by an individual (i.e. dominant or high social status versus subordinate or low social status) can impact its physiological status. For example, within pairs of juvenile salmonids, dominant fish tend to exhibit a transient increase in circulating levels of catecholamine (Thomas and Gilmour, 2006) and cortisol [the main glucocorticoid (GC) in teleosts; Øverli et al., 1999] stress hormones during the initial period of hierarchy formation. Dominant fish also exhibit longer-term benefits from the monopolization of food (e.g. Metcalfe et al., 1989; McCarthy et al., 1992; Øverli et al., 1998), and changes such as increases in liver glycogen stores (Gilmour et al., 2012) and transcript levels of cortisol steroidogenic genes (Jeffrey et al., 2012) that suggest an enhanced ability to deal with subsequent stressors (‘pre-adaptation’). Subordinate fish, in contrast, experience chronic social stress, as indicated by sustained elevation of circulating cortisol levels accompanied by reduced reactivity to additional stressors (Jeffrey et al., 2014), reduced growth rates, which also reflect low food intake and changes in digestive function (DiBattista et al., 2006), and increased standard metabolic rate (Sloman et al., 2000). Thus, there are negative impacts of low social status, but also potential benefits for those individuals that maintain dominant status. Whether these differences at the individual level translate into consequences for subsequent generations remains understudied, in part owing to challenges posed by the life history of salmonid fish.

Zebrafish (Danio rerio) provide a useful species in which to study the potential impacts of social status on offspring. Adult zebrafish form social hierarchies, with one fish becoming dominant through aggressive interactions (Larson et al., 2006; Filby et al., 2010; Paull et al., 2010; Dahlbom et al., 2011; Pavlidis et al., 2011). The physiological consequences of social stress have been evaluated to some extent in zebrafish. Dominant fish tended to be larger (Filby et al., 2010; Paull et al., 2010), and dominant males exhibited a higher growth rate compared with subordinate fish (Filby et al., 2010). As in salmonids, subordinate zebrafish exhibited elevated cortisol levels and subordinate males displayed higher telencephalic corticotropin-releasing factor (crf) and glucocorticoid receptor (gr) mRNA levels on the first day of interaction (Filby et al., 2010). Furthermore, preoptic area (POA) levels of arginine vasotocin (AVT), a mediator of social behaviour and stress axis activity, differed between dominant and subordinate fish (Larson et al., 2006). Subordinate fish also exhibited increased brain serotoninergic activity as indicated by elevated hindbrain 5-hydroxyindoleacetic acid (5-HIAA) to serotonin (5-hydroxytryptamine, 5-HT) ratios following 5 days of interaction (Dahlbom et al., 2012), and social status influenced reproductive success and mate choice (Paull et al., 2010). Together, these studies clearly display the impacts of low social status or chronic social stress in adult zebrafish. The goal of the present study, then, was to determine whether social stress experienced by an adult female zebrafish has consequences for her offspring, specifically with respect to development of the stress response.
Maternal stress has been reported to affect egg quality (e.g. Campbell et al., 1992, 1994; Giesen et al., 2011; Mileva et al., 2011), as well as the size, physiology and behaviour of offspring (e.g. McCormick, 1998, 2006, 2009; Giesen et al., 2011; McGhee et al., 2012; Roche et al., 2012; Mommer and Bell, 2013; Eaton et al., 2015; Sopinka et al., 2015). Maternally derived cortisol, which is deposited to oocytes around the time of vitellogenesis, has been suggested as a potential mediator of the effects of maternal stress on offspring. Maternal cortisol plays an important organizational role during early development in fish, and cortisol treatment of eggs or embryos alters both physiology and behaviour of the developing young (e.g. Sloman, 2010; Burton et al., 2011; Li et al., 2012; Nesan and Vijayan, 2012; Colson et al., 2015; Sopinka et al., 2015). However, programming of offspring is probably more complex than the action of cortisol alone, because previous studies have found effects of maternal stress even in the absence of increased embryo cortisol levels – for example, on egg size in Neolamprologus pulcher (Mileva et al., 2011) and on swim performance in Oncorhynchus nerka (Sopinka et al., 2014).

Ontogeny of the hypothalamic-pituitary-interrenal (HPI) axis has been well studied in zebrafish (Alsop and Vijayan, 2008; Alderman and Bernier, 2009; Wilson et al., 2013), providing the necessary groundwork for investigating effects of maternal social stress. Specific maternal mRNAs associated with the HPI axis are transferred to eggs and play a key role in embryogenesis before embryos begin to synthesize mRNAs (i.e. become transcriptionally active). Transcripts for crf, its binding protein and its receptors are present in eggs; however, their role in early development remains unclear (Alderman and Bernier, 2009). Maternal gr transcripts mediate cortisol-dependent actions during early development (Nesan and Vijayan, 2013). In addition, cytochrome P450 side-chain cleavage enzyme (P450scc)-synthesized pregnenolone is involved in early embryogenesis and epiboly (Hsu et al., 2009). As embryos become transcriptionally active, mRNA abundance of HPI axis genes increases (Alsop and Vijayan, 2009). At this point, zebrafish larvae possess all of the machinery necessary to synthesize cortisol de novo, and by 72 h post-fertilization (hpf), they begin to respond to stressors with an increase in cortisol levels (Alderman and Bernier, 2009; Wilson et al., 2013).

The present study tested the hypothesis that social status would influence the maternal contribution of cortisol and HPI axis-related mRNA to offspring (measured at 1 hpf), offspring cortisol levels and mRNA abundance of HPI axis genes over development, and the larval stress response. Previous studies reported that stressed female fish gave rise to offspring with a dampened cortisol response to a stressor (Sopinka et al., 2015), and that embryos exposed to cortisol exogenously exhibited a dampened stress response at later stages of development (Auperin and Geslin, 2008). Therefore, a similar attenuation of the stress response was predicted to occur in offspring of subordinate mothers experiencing chronic social stress. To investigate the mechanisms underlying such an effect, maternal contributions to offspring and mRNA abundance of key stress axis genes were examined during early development across offspring of sham-treated (females handled in the same way as paired fish, but not paired with a conspecific), dominant and subordinate fish.

**MATERIALS AND METHODS**

**Experimental animals**

Adult zebrafish (N=183; 0.588±0.015 g, mean±s.e.m.), purchased from AQUAlity Tropical Fish Wholesale in Mississauga, ON, Canada, were housed under a 14 h light:10 h dark photoperiod in 3 and 10 l flow-through polycarbonate tanks supplied with aerated, dechlorinated water from Ottawa tap water at 28°C. Fish were fed once or twice daily to satiation with No. 1 crumble-Zeigler (Aquatic Habitats, Apopka, FL, USA) as well as brine shrimp. Fish were acclimated to these holding conditions for at least 3 weeks prior to experimentation. Embryos were reared in 50 ml Petri dishes containing embryo medium (0.01% Methylene Blue, NaCl 0.275 g l⁻¹, KCl 0.012 g l⁻¹, MgSO₄·7H₂O 0.078 g l⁻¹, CaCl₂·2H₂O 0.046 g l⁻¹) and kept in a 28°C incubator. All experiments were conducted in compliance with the guidelines of the Canadian Council on Animal Care (CCAC) and after approval from the University of Ottawa Animal Care Committee (protocol BL-280).

Female adult zebrafish were tagged with a unique mark using Alcian Blue (Sigma-Aldrich, Oakville, ON, Canada). Fish were lightly anaesthetized in a buffered solution of MS-222 (0.24 mg ml⁻¹; 3-amino benzoic acid ethyl ester; 21 mmol l⁻¹ Tris, pH 7; Sigma-Aldrich; Westerfield, 2000), blotted dry, weighed, measured for fork length, and marked. Preliminary experiments revealed that fish were less likely to provide good quality embryos following marking and social stress, a problem that was improved by allowing fish to recover for 2 days in their original tank prior to being paired. Fork length-matched females were then placed in pairs (N=74; difference in fork length 1.25±0.15%) or on their own for the sham treatment (N=35) in experimental tanks (4.5 l). A sham group was included in the experimental design to account for the potential effects of handling and other stressors (e.g. transfer to the experimental tank) experienced by fish over the course of the experiment. Fish were placed individually on either side of an opaque perforated divider (day 1) and on the morning of day 2, the divider was removed, allowing fish to interact. Each fish within a pair was observed twice daily for 3 min each time on days 2 and 3. Fish were assessed for position within the water column, feeding (first to feed), acts of aggression, and retreats/freezing behaviour. Dominant behaviours (acts of aggression and monopolizing of food and territory) received higher scores than subordinate behaviours (retreats and freezing). The fish within a pair with the higher overall score was assigned dominant status. Previous studies have used similar scoring systems (Filby et al., 2010; Paull et al., 2010). An interaction period of 48 h was chosen to maximize social interactions while minimizing decrements in physiological condition in subordinate fish that might impair reproduction. Preliminary experiments indicated that moving females to a breeding chamber with a male (separated from the male by a clear divider) prior to sampling improved the success of retrieving viable eggs from the females. Females were terminally anaesthetized in a buffered solution of MS-222 (as above but using 0.72 mg ml⁻¹) on the morning of day 4, weighed and stripped of their eggs before being flash frozen in liquid nitrogen. Each female’s eggs were collected into a 50 ml Petri dish and fertilized with sperm, pooled

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**List of abbreviations**

- 11β-HSD2: 11β-hydroxysteroid dehydrogenase type 2
- 11β-hyd: 11β-hydroxylase
- crf: corticotropin-releasing factor
- crf-r: corticotropin-releasing factor receptor
- GC: glucocorticoid
- HPA: hypothalamic-pituitary–adrenal
- hpf: hours post-fertilization
- HPI: hypothalamic-pituitary–interrenal
- P450scc: cytochrome P450 side chain cleavage enzyme
- POA: preoptic area
- S-QPCR: semi-quantitative real-time RT-PCR
- star: steroidogenic acute regulatory protein
from 11–20 males, collected in Hanks’ solution (Sigma-Aldrich) (see Westerfield, 2000, for similar methods), and Petri dishes were placed in a 28°C incubator.

All embryos were counted to determine clutch size, where clutch size refers to the number of eggs stripped from the female. Clutches were reared to 1, 24, 48, 96 or 144 hpf. At each time point, groups of 25 embryos or larvae were collected for cortisol analysis, and groups of 100 (1 hpf) to 15 (144 hpf) embryos/larvae were collected for analysis of mRNA abundance; collected embryos were flash frozen in liquid nitrogen and stored at −80°C. A female’s clutch constituted a single N for a time point.

After fertilization, embryo volume was determined using ImageJ (http://imagej.nih.gov/ij/) from pictures taken using a Micropublisher 3.3 RTV camera (Q Imaging, Surrey, BC, Canada) and an Olympus SZ61 dissecting microscope. The equation for an ellipsoid was used to estimate embryo volume. Survival to 24 hpf, hatching success (at 48 hpf) and survival over the developmental period were also assessed.

Maternal cortisol levels
Cortisol was extracted from a subset (N=12 per treatment group) of adult female zebrafish using a protocol adapted from Fuzzen et al. (2010, 2011). In brief, zebrafish were powdered in liquid nitrogen with a mortar and pestle prior to homogenization with a handheld operated pestle grinder (Kimble Chase Kontes, Rockwood, TN, USA). Samples were extracted three times with 1 ml of methanol each time. After each addition of methanol, samples were vortexed thoroughly and incubated at 4°C in the dark for 60 min (for the first extraction) or 30 min (for the second and third extractions). Samples were centrifuged at 3000 g for 5 min at 4°C and flash frozen at −80°C for 10 min. After each extraction, the supernatant was transferred to a 2 ml microtube and evaporated under forced air. Combined extractions were reconstituted in 2 ml of acetate buffer (2.35 ml glacial acetic acid and 1.23 g sodium acetate trihydrate per litre). Reconstituted samples were purified by passing them through a 500 mg C18 solid phase extraction column (Fisher Scientific) primed with methanol and milliQ water. Prior to elution, ultra-pure water (Caymen Chemical, Cedarlane, Burlington, ON, Canada) and hexane were passed through the column. Steroids were eluted slowly from the column four times with 1 ml of ethyl acetate (1% methanol) each time. The eluates were combined into a 2 ml microtube, dried under forced air and then reconstituted in 1 ml of extraction buffer [from Neogen cortisol enzyme-linked immunoassay (EIA) kit, Lexington, KY, USA]. Samples were heated at 60°C for 5 min, vortexed twice, and stored at −80°C until analysis by EIA (Neogen). Samples were diluted 2-fold and assayed in duplicate on a single plate where intra-assay variability was 5.6%. Extraction efficiency was determined by spiking homogenates with a known amount of 3H-hydrocortisone and was 59%.

Embryo/larval baseline and stress-induced cortisol levels
Baseline, whole-body cortisol levels were assessed at all time points, whereas stress-induced cortisol levels in response to a ‘swirling’ stressor were determined at 96 and 144 hpf according to Alsop and Vijayan (2008). Cortisol was extracted from pooled embryos/larvae using a protocol modified from Sopinka et al. (2014). In brief, samples were partially thawed on ice, and homogenized in 200 μl of extraction buffer (Neogen) in a 1.5 ml microtube using a battery-operated pestle grinder (Kimble Chase Kontes, Rockwood, TN, USA). Samples were extracted three times with 1 ml of diethyl ether (Fisher Scientific) each time. After each addition of diethyl ether, samples were vortexed thoroughly, incubated at room temperature for 30 min (15 min for the second and third extractions), centrifuged at 3000 g for 5 min, and flash frozen at −80°C for 30 min. The liquid

Table 1. Oligonucleotide primer sets for semi-quantitative real-time RT-PCR in zebrafish (Danio rerio)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5′-3′)</th>
<th>Accession no.</th>
<th>Reference*</th>
</tr>
</thead>
<tbody>
<tr>
<td>crf</td>
<td>F: gcc gcg caa agt tca aaa</td>
<td>BC085458</td>
<td>Alsop and Vijayan, 2008</td>
</tr>
<tr>
<td></td>
<td>R: gcggagaaccttgtgcgtaaa</td>
<td>XM691254</td>
<td>Alsop and Vijayan, 2008</td>
</tr>
<tr>
<td>crf-r1</td>
<td>F: gaa atg cca cct ggt tcc tg</td>
<td>ENSDART 00000055713</td>
<td></td>
</tr>
<tr>
<td>crf-r2</td>
<td>R: agg ctc cac cag atc aca tt</td>
<td>NM131663</td>
<td></td>
</tr>
<tr>
<td>star</td>
<td>F: tgt ggg aat gga cat cgc tc</td>
<td>NM152953</td>
<td>Alderman and Bernier, 2009</td>
</tr>
<tr>
<td>p450sc</td>
<td>R: cac tgt tgc tct cgg ca</td>
<td>NM001080204</td>
<td>Ings and Van Der Kraak, 2006</td>
</tr>
<tr>
<td>11β-hyd</td>
<td>F: gct cat gca cat tct gag ga</td>
<td>NM212720</td>
<td></td>
</tr>
<tr>
<td>11β-hsd2</td>
<td>R: tgt gct gaa gat gct cag tg</td>
<td>NM001020711</td>
<td>Alsop and Vijayan, 2008</td>
</tr>
<tr>
<td>gr</td>
<td>F: gcc att gag cac caa atc ac</td>
<td>EFS67113</td>
<td>Alsop and Vijayan, 2008</td>
</tr>
<tr>
<td>mr</td>
<td>R: agt aga gca ttt ggg cgt tg</td>
<td>FJ915075</td>
<td></td>
</tr>
<tr>
<td>18s</td>
<td>F: ggc gcc gtt att ccc atg acc</td>
<td>AF025305</td>
<td>Alsop and Vijayan, 2008</td>
</tr>
<tr>
<td>β-actin</td>
<td>R: ggt gtt gct ccc tcc tca att c</td>
<td>BC105746</td>
<td>Alsop and Vijayan, 2008</td>
</tr>
<tr>
<td>ubiquitin</td>
<td>F: tgt ccc tgt atg cct ctg gt</td>
<td>ENSDART</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: aag tcc aga cgg aga atg g</td>
<td>BC085458</td>
<td>Alsop and Vijayan, 2008</td>
</tr>
</tbody>
</table>

*Where no reference is provided, primers were designed for the present study using Primer3 or Primer3Plus, and primer specificity was verified by sequencing (GenScript, Piscataway, NJ, USA). crf, corticotropin-releasing factor; crf-r, crf receptor; star, steroidogenic acute regulatory protein; 11β-hyd, 11β-hydroxylase; 11β-hsd2, 11β-hydroxysteroid dehydrogenase 2; gr, glucocorticoid receptor; mr, mineralocorticoid receptor.
Phases of each extraction were combined, transferred to a clean 1.5 ml microtube, and evaporated under forced air. The extract was reconstituted in 250 μl of extraction buffer (Neogen), vortexed, heated at 65°C for 5 min twice, and stored at −80°C until analysis by EIA (Neogen). Samples were assayed in duplicate over four plates where inter-assay variability was 3.1% and intra-assay variability was 7.8%. Because solid phase extraction was not used, a subset of samples was used to generate dilution curves that were found to be parallel to the standard curve. Extraction efficiency was determined by spiking homogenates with a known amount of 1H-hydrocortisone and was 85% for embryos at 1 hpf and 79% for larvae at 144 hpf.

Embryo mRNA abundance
Total RNA was extracted from pools of 15–100 embryos/larvae using TRIzol reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer’s protocol. Embryos/larvae were homogenized in 0.5 ml of TRIzol reagent using a battery-operated pestle grinder (Kimble Chase Kontes). Extracted RNA was quantified using a NanoDrop® ND-2000c UV-Vis Spectrophotometer (Thermo Scientific, Ottawa, ON, Canada). Next, cDNA was synthesized from 0.5 μg of RNA using QuantiTect Reverse Transcription Kit (Qiagen, Toronto, ON, Canada) and following the manufacturer’s protocol with the exception that half reaction volumes, with a final extraction buffer (Neogen), vortexed, heated at 65°C for 5 min twice, and stored at 80°C until analysis by

Table 2. Clutch size and offspring viability (i.e. embryo volume, survival at 24 hpf and hatching at 48 hpf) for sham-treated, dominant and subordinate female zebrafish (Danio rerio)

<table>
<thead>
<tr>
<th>Maternal social status</th>
<th>Clutch size (μg)</th>
<th>Embryo volume (at 1 hpf, mm³)</th>
<th>Survival to 24 hpf (%)</th>
<th>Hatching at 48 hpf (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>353.0±28.9</td>
<td>0.0729±0.003</td>
<td>81.7±3.8</td>
<td>52.3±6.8</td>
</tr>
<tr>
<td>Dominate</td>
<td>363.2±24.3</td>
<td>0.0738±0.005</td>
<td>70.1±4.7</td>
<td>41.9±6.4</td>
</tr>
<tr>
<td>Subordinate</td>
<td>333.5±30.8</td>
<td>0.0767±0.007</td>
<td>68.5±5.0</td>
<td>37.1±6.6</td>
</tr>
</tbody>
</table>

Fig. 1. Survival of offspring of sham-treated (N=8), dominant (N=12) and subordinate (N=13) female zebrafish (Danio rerio) over 1–144 h post-fertilization (hpf). Values are presented as means±s.e.m. No significant effect of maternal treatment was found. Time points that do not share a letter are significantly different from one another (two-way RM ANOVA; P=0.475 for maternal status, P<0.001 for time point, P=0.546 for treatment×time).

Statistical analysis
Cortisol concentrations and mRNA abundance of HPI axis genes have been well characterized throughout development in zebrafish (Alsop and Vijayan, 2008; Alderman and Bernier, 2009; Wilson normalize Ct values. Normalized Ct values were then expressed relative to the sham 1 hpf mean [for crf, crf-receptor 1 (crf-r1), crf-r2, p450sc and gr], the sham 24 hpf mean [for steroidogenic acute regulatory protein (star), 1β-hydroxylase (1β-hyd) and mineralocorticoid receptor (mr)] or the sham 48 hpf mean (for 11β-hsd2) using the −ΔCt method (Livak and Schmittgen, 2001). Genes were expressed relative to a group other than the sham 1 hpf when expression levels for the gene were undetectable at 1 hpf.

Fig. 2. Baseline whole-body cortisol levels for offspring of sham-treated (N=5–6), dominant (N=6–8) and subordinate (N=6–8) female zebrafish over 1–144 hpf. Independent samples were pools of 25 offspring from a single female. Values are expressed per individual offspring and presented as means±s.e.m. Maternal status effects were analysed by one-way ANOVA (on ranks indicated by italics, and significant values are in bold). Within a time point, groups that do not share a letter are significantly different from one another.
Another time point, groups that do not share a letter are significantly different from one another. Within a time point, only maternal effects within a developmental time point were evaluated statistically. The level of significance (α) was 0.05. All analyses were carried out using JMP V.11.

**RESULTS**

**Effects of maternal social status on egg production and early embryo development**

Maternal whole-body cortisol levels measured at the end of the experiment did not differ with social status (sham-treated, 7.74±1.31 ng g⁻¹ body mass; dominant, 8.03±1.40 ng g⁻¹ body mass; subordinate, 6.17±1.29 ng g⁻¹ body mass; one-way ANOVA, P=0.574). Both dominant (56.8%; N=74) and subordinate

**Fig. 3.** Relative mRNA abundance of corticotropin-releasing factor, crf-receptor 1 and crf-r2 in offspring of sham-treated (N=5–6), dominant (N=6) and subordinate (N=4–6) female zebrafish over 1–144 hpf. (A) corticotropin-releasing factor (crf); (B) crf-receptor 1 (crf-r1); and (C) crf-r2. Levels of mRNA were measured by semi-quantitative real-time RT-PCR using 15–100 pooled offspring from a single female as a single N. Values are presented as means±s.e.m. All data were expressed relative to the 1 hpf group from sham-treated females. Maternal status effects were analysed by one-way ANOVA (on ranks indicated by italics, and significant values are in bold). Within a time point, groups that do not share a letter are significantly different from one another.

**Fig. 4.** Relative mRNA abundance of steroidogenic acute regulatory protein, cytochrome p450 side chain cleavage enzyme and 11β-hydroxylase in offspring of sham-treated, dominant and subordinate female zebrafish over 1–144 hpf. (A) steroidogenic acute regulatory protein (star); (B) cytochrome p450 side chain cleavage enzyme (p450scc); and (C) 11β-hydroxylase (11β-hyd). Levels of mRNA were measured by semi-quantitative real-time RT-PCR using 15–100 pooled offspring from a single female as a single N. Values are presented as means±s.e.m. (N=5–6 for all groups). Data were expressed relative to the 24 hpf group from sham-treated females for star and 11β-hyd, and relative to the 144 hpf group from sham-treated females for p450scc. Maternal status effects were analysed by one-way ANOVA (on ranks indicated by italics, and significant values are in bold). Within a time point, groups that do not share a letter are significantly different from one another.
from one another. Within a time point, groups that do not share a letter are significantly different (one-way ANOVA; on ranks indicated by italics, and significant values are in bold).

Fig. 5. Relative mRNA abundance of glucocorticoid receptor, mineralocorticoid receptor and 11β-hydroxysteroid dehydrogenase type 2 in offspring of sham-treated (N=5–6), dominant (N=5–6) and subordinate (N=6) female zebrafish. Data are shown over 1–144 hpf for (A) glucocorticoid receptor (gr) and (B) mineralocorticoid receptor (mr); and over 48–144 hpf for (C) 11β-hydroxysteroid dehydrogenase type 2 (11β-hsd2, see Materials and methods). Levels of mRNA were measured by semi-quantitative real-time RT-PCR using 15–100 pooled offspring from a single female as a single N. Values are presented as means+s.e.m. Data were expressed relative to 1 hpf offspring from sham-treated females for gr, relative to 24 hpf offspring from sham-treated females for mr, and relative to 48 hpf offspring from sham-treated females for 11β-hsd2. Maternal status effects were analysed by one-way ANOVA (on ranks indicated by italics, and significant values are in bold). Within a time point, groups that do not share a letter are significantly different from one another.

(59.5%; N=74) females yielded viable clutches less often than sham-treated females (91.4%; N=35; Pearson’s χ² test, P=0.010). Maternal social status had no significant effect on clutch size or early embryo viability (i.e. survival, embryo volume at 1 hpf, hatching at 48 hpf; Table 2, Fig. 1).

Offspring baseline cortisol levels and mRNA abundance of HPI axis genes

Baseline whole-body cortisol levels (Fig. 2) and mRNA abundance of HPI axis genes (Figs 3–5) were unaffected by maternal treatment until 48 hpf. At 48 hpf, cortisol levels were significantly lower in offspring of dominant females compared with those of subordinate females (Fig. 2). Similarly, mRNA abundance of crf and p450scc was significantly lower at 48 hpf in offspring of dominant females compared with offspring of subordinate and sham-treated females, respectively (Figs 3A and 4B).

Although baseline cortisol levels were unaffected by maternal social status after 48 hpf (Fig. 2), mRNA abundance of HPI axis genes continued to be modulated by maternal status. At 96 hpf, crf mRNA abundance tended to be lower in offspring of dominant females (Fig. 3A). Similarly, transcript levels of star (Fig. 4A) and mr (Fig. 5B) were significantly lower in offspring of dominant females than in offspring of sham-treated or subordinate females at 96 hpf. Conversely, crf-r2 mRNA levels were elevated in offspring of subordinate females compared with offspring of sham-treated or dominant females at 96 hpf (Fig. 3C). At 144 hpf, crf mRNA abundance tended to be elevated (Fig. 3A) and crf-r1 mRNA abundance was significantly elevated in offspring of dominant females (Fig. 3B). Likewise, maternal dominance resulted in
Offspring stress-induced cortisol levels

Cortisol levels were measured 5 min after exposure to a 30 s swirling stressor (stressed) for groups of 25 larvae at 96 and 144 hpf (Fig. 6). At 96 hpf, only larvae of subordinate females exhibited significantly elevated cortisol levels (Fig. 6A; paired Student’s t-test; P=0.009), overall, however, stressed cortisol levels did not differ significantly with maternal treatment at 96 hpf (Fig. 6A; one-way ANOVA; P=0.426). At 144 hpf, all larvae significantly elevated cortisol levels in response to the swirling stressor (Fig. 6B; paired Student’s t-tests; P=0.003, 0.006 and 0.031 for offspring of sham-treated, dominant and subordinate females, respectively), but stress-induced cortisol levels were significantly lower in larvae of subordinate females compared with offspring of sham females (Fig. 6B; one-way ANOVA; P=0.030).

DISCUSSION

Effects of maternal social status on egg production and early embryo development

Social stress did not affect whole-body cortisol levels in the adult female zebrafish of the present study. Filby et al. (2010) reported that cortisol levels were elevated as a result of low social status for zebrafish held in groups of four fish (two females and two males per group). However, Pavlidis et al. (2011) found no difference in whole-trunk cortisol levels between dominant and subordinate zebrafish after 5 days of interaction, although the levels in both dominant and subordinate fish were elevated above those of control fish. In the present study, female fish were moved to breeding tanks more than 12 h prior to sampling (see Materials and methods) and this time may have been sufficient for social status-induced differences in cortisol levels to be masked by cortisol metabolism. The metabolic clearance of cortisol depends on the rate of tissue uptake and steroid catabolism (Mommsen and Walsh, 1988), which do not appear to have been measured in zebrafish. However, Faught et al. (2016) reported that a bolus of cortisol administered via food intake was not detectable after 12 h, suggesting that cortisol clearance is rapid in this species. Elevation of cortisol levels is not, however, the only consequence of social interactions or social stress.

Competition for dominance requires fish to invest energy in agonistic contests (Johnsson et al., 2006), and thus energy may have been reallocated from reproduction to social contests, resulting in dominant and subordinate females exhibiting viable clutches less often than sham-treated females (where clutch refers to the eggs stripped from the female). For the viable clutches obtained, maternal social status had no apparent effect on clutch size or offspring viability during early development (i.e. embryo size, survivability or hatching at 48 hpf). However, the possibility that females of different social status could produce clutches of different size or at different frequencies if allowed to spawn naturally cannot be excluded. The results of previous studies have been mixed, with evidence for maternal stress effects on offspring size and survival in rainbow trout (Oncorhynchus mykiss) and/or brown trout (Salmo trutta) (Campbell et al., 1994; Contreras-Sánchez et al., 1998), damselfish (Pomacentrus amboinensis) (McCormick, 2006), Neolamprologus pulcher (Mileva et al., 2011) and three-spined stickleback (Gasterosteus aculeatus) (Giesing et al., 2011), but no effect on gamete quality in coho salmon (Oncorhynchus kisutch) (Stratholt et al., 1997), or on egg size or early offspring survival in sockeye salmon (O. nerka) (Sopinka et al., 2014). This variability may reflect, at least in part, differences in the severity of the stressor experienced by the female fish; in the present study, subordinate social stress may not have been sufficiently severe to induce effects beyond those resulting from the demands of social interaction. Moreover, the timing of the stressor relative to oocyte development may play a role in determining the quality of the offspring. Zebrafish are asynchronous breeders and therefore social stress would have occurred during the final stages of oocyte development for the eggs examined in the present study.

Maternal social status affected cortisol and HPI axis function during early development

Egg (1 hpf embryo) cortisol levels were not affected by social status in the present study. In fish, maternal cortisol contribution to the egg occurs around the time of vitellogenesis (Selman et al., 1993; Mommsen et al., 1999). In asynchronously breeding zebrafish, cortisol deposition to the developing oocyte is actively controlled through ovarian 11β-HSD-2 activity and probably occurs only during a short window (Faught et al., 2016). Consequently, increases in embryo cortisol levels as a result of maternal stress will not be visible in zebrafish embryos until about 9 days after the period of maternal stress (Faught et al., 2016), i.e. after the period of egg collection used in the present study. Even in the absence of increased maternal cortisol transfer to embryos, Sopinka et al. (2014) reported that maternal stress caused differences in offspring swim performance, suggesting that cortisol is not the only mediator of offspring programming by maternal stress. Similarly, the present study found evidence of effects of maternal social status on development of the HPI axis in zebrafish offspring without differences in maternal contribution of cortisol or mRNA levels to eggs, suggesting that maternal social status may affect oocytes even during their late stages of maturation. These effects were first observed at 48 hpf, the time of hatching and the point in development at which zebrafish begin to synthesize cortisol de novo.

De novo synthesis of cortisol and activation of the stress response are delayed in teleost fish until after hatch (Alsop and Vijayan, 2008; Alderman and Bernier, 2009). At 48 hpf, offspring of dominant females exhibited lower baseline cortisol levels than offspring of subordinate females. Correspondingly, mRNA levels of both crf and p450scc were lower in offspring of dominant females. Lower cortisol levels, perhaps owing to lower mRNA levels of these key HPI axis genes, might reflect a delay in the de novo synthesis of cortisol in offspring of dominant females. Cortisol affects hatching rate in zebrafish and blocking cortisol synthesis either by morpholino knockdown of 11β-hyd or with metyrapone (an 11β-hyd enzyme inhibitor) decreased the proportion of embryos hatched by 72 hpf (Wilson et al., 2013). The lower baseline cortisol levels in embryos of dominant females did not appear to delay hatching rate in the present study. However, hatching was measured only at 48 hpf, which may have hindered the detection of differences; Wilson et al. (2013) detected significant effects of metyrapone and cyp11b1 knockdown at 72 but not 48 hpf. It is interesting that this impact of maternal status on offspring cortisol levels during an important point in development was associated with dominant rather than subordinate social status. It adds another instance where high social status has distinct physiological consequences (for other examples, see Gilmour et al., 2012; Jeffrey et al., 2012) and emphasizes the importance of considering maternal environment and experience broadly in examining effects on offspring.
Effects of maternal social status on HPI axis genes were also observed at 96 and 144 hpf, even though offspring baseline cortisol levels did not differ significantly with maternal treatment at these time points. Similar to the results at 48 hpf, crf mRNA abundance tended to be lower in offspring of dominant females at 96 hpf, as were mRNA levels of star, effects with the potential to lower cortisol levels; the absence of any impact on cortisol levels suggests either compensation by other elements of the stress axis or a mismatch between mRNA and protein levels. Interestingly, by 144 hpf, crf mRNA abundance tended to be elevated and crf-r1 transcript levels were significantly so in offspring of dominant females, which might have been expected to increase baseline cortisol levels. However, transcript levels of the cortisol breakdown enzyme 11β-HSD2 also were significantly elevated in these offspring, which may account for the absence of an effect on baseline cortisol. Together, these results suggest that maternal dominance affected baseline expression of genes associated with stress axis function during early points in development, even in the absence of changes in baseline cortisol levels beyond 48 hpf. The mechanisms through which maternal social status late in oocyte development affect stress axis development remain to be determined. In addition, the question of whether these effects of maternal dominance have functional consequences for older offspring warrants investigation.

**Cortisol production in response to a stressor is affected by maternal social status**

Larvae exhibited a robust cortisol response to a swirling stressor only at 144 hpf. Although stress-induced elevation of cortisol has been observed as early as 72 hpf in zebrafish larvae (Alderman and Bernier, 2009; Wilson et al., 2013), these studies used different, perhaps more severe, stressors than the swirling stressor used in the present study. Notably, Alsop and Vijayan (2008) using the same swirling stressor detected an acute cortisol response only at 97 hpf (in their study, fish were not tested beyond 97 hpf). No effect of maternal social status was detected at 96 hpf in the present study, although it is possible that assessing the cortisol response over a longer time scale (i.e. beyond 5 min post-stressor) might have revealed differences in the dynamics of the stress response that were not captured in the present study (e.g. Nesan and Vijayan, 2016). Stressor-induced cortisol levels were significantly lower in the offspring of subordinate females at 144 hpf. This apparent plasticity of the HPI axis during early development also has emerged in other studies. For example, developing rainbow trout exposed to either a stressor (air exposure/cold water) or cortisol treatment during early development (Auperin and Geslin, 2009) and sockeye salmon reared from mothers subjected to a chasing stressor (Sopinka et al., 2008) revealed differences in the dynamics of the stress response that were not captured in the present study (e.g. Nesan and Vijayan, 2016). Stressor-induced cortisol levels were significantly lower in the offspring of subordinate females at 144 hpf. This apparent plasticity of the HPI axis during early development also has emerged in other studies.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

J.D.J. and K.M.G. contributed equally to the experimental design, analysis and writing of the manuscript. J.D.J. performed all experiments.

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