MODULATION OF STRESS RESPONSES BY FKBP5 IN ZEBRAFISH AND RAINBOW TROUT

Donna St-Amant

Thesis submitted to the University of Ottawa
in partial fulfillment of the requirements for the
Master's degree in biology

Department of Biology
Faculty of Science
University of Ottawa

© Donna St-Amant, Ottawa, Canada, 2026

Abstract

Environmental challenges activate the hypothalamic-pituitary-interrenal (HPI) axis in fishes, increasing synthesis of the glucocorticoid stress hormone cortisol. Cortisol binds to the glucocorticoid receptor (GR), which activates negative feedback regulation of the HPI axis. In mammals, activation of GR increases FK506-binding protein 51 (FKBP5), a co-chaperone of GR that inhibits its activity. The present study hypothesized that FKBP5 and GR interactions similarly contribute to prolonged elevation of cortisol during chronic stress in rainbow trout (*Oncorhynchus mykiss)*. Subordinate trout exhibited elevated plasma cortisol, and after 4 d of social interaction, also displayed elevated transcript abundance of *fkbp5B*. Treatment of subordinates with an FKBP5 inhibitor, SAFit2, tended to lower cortisol but the effects were not clear cut. The expression of *fkbp5* in zebrafish (*Danio rerio*) was assessed during early development and in response to an acute stressor in both wildtype fish and those lacking the GR (GR-KO). The GR-KO fish exhibited significantly lower *fkbp5* expression in both larvae and adults compared to wildtype fish. The transcript abundance of *fkbp5* exhibited transient elevation in response to an acute stressor in wildtype fish. Overall, my findings suggest that FKBP5 plays a role in maintaining elevated cortisol during chronic stress in rainbow trout and show that GR regulates the baseline expression of *fkbp5* across development in zebrafish.

Résumé

Les stress environnementaux activent l'axe hypothalamo-hypophyso-surrénalien (HPI) chez les poissons, entraînant une augmentation de la synthèse du cortisol, une hormone de stress glucocorticoïde. Le cortisol se lie au récepteur des glucocorticoïdes (GR), ce qui active la régulation par rétroaction négative de l'axe HPI. Chez les mammifères, l'activation du GR augmente la protéine 51 de liaison au FK506 (FKBP5), une co-chaperonne du GR qui inhibe son activité. La présente étude a émis l'hypothèse que les interactions entre la FKBP5 et le GR contribuent de manière similaire à une élévation prolongée du cortisol lors d'un stress chronique chez la truite arc-en-ciel (*Oncorhynchus mykiss*). Les truites subordonnées présentaient un taux élevé de cortisol plasmatique et, après 4 jours d'interaction sociale, affichaient également une abondance élevée de transcrits de *fkbp5B*. Le traitement des subordonnés avec un inhibiteur de la FKBP5, le SAFit2, a eu tendance à réduire le cortisol, mais les effets n'étaient pas clairement établis. L'expression de *fkbp5* chez le poisson zèbre (*Danio rerio*) a été évaluée au cours du développement précoce et en réponse à un facteur de stress aigu, tant chez les poissons de type sauvage que chez ceux dépourvus de GR (GR-KO). Les poissons GR-KO présentaient une expression de *fkbp5* significativement plus faible, tant chez les larves que chez les adultes, par rapport aux poissons de type sauvage. L'abondance des transcrits de *fkbp5* a présenté une élévation transitoire en réponse à un facteur de stress aigu chez les poissons de type sauvage. Dans l'ensemble, mes résultats suggèrent que FKBP5 joue un rôle dans le maintien d'un taux élevé de cortisol pendant le stress chronique chez la truite arc-en-ciel et montrent que le GR régule l'expression de base de *fkbp5* tout au long du développement.

Acknowledgments

First are foremost, I would like to thank Dr. Katie Gilmour for her patience, advice, and understanding throughout the past four years. She never hesitated to answer my questions each time I knocked on her door and guided me through each step of my time in the lab with such kindness. I also want to thank my advisory committee Dr. Marc Ekker and Dr. Jan Mennigen, for their encouragement, questions, and ideas for this project. Thank you to Vishal Saxena for initiating the *fkbp5*-KO line and supplying us with F_1 fish.

Thank you to each member of the Gilmour lab for their help throughout each stage of this project. I would like to thank Dr. Brittany Bard for teaching me all about rainbow trout and RT-PCR. Thank you to Dr. Patrícia Ferreira for her willingness to answer my questions and help troubleshoot, especially her guidance in developing the *fkbp5*-KO line. I would also like to thank Patrícia for her work on the rainbow trout tissue distribution. Thank you to Michael Tea for measuring baseline cortisol values of the GR-KO and mWT fish. Thank you to Kaitlyn Flear for her help with the tricky hypoxia set up. Finally, thank you to Hannah Stairs for her support as we both searched for heterozygous fish.

Lastly, I'd like to thank my family and friends for their continued support during this process.

Table of Contents

Abstract ..... **ii**

Résumé ..... **iii**

Acknowledgements ..... **iv**

List of tables and figures ..... **vii**

List of abbreviations ..... **xi**

Chapter 1: General introduction **1**

1.1 Thesis overview 2

1.2 The stress axis 2

1.3 GR and FKBP5 5

1.4 Hypotheses and objectives 9

Chapter 2: A role for FKBP5 in modulating cortisol levels in subordinate rainbow trout **11**

2.1 Introduction 12

2.2 Materials and methods 15

2.2.1 Experimental animals 15

2.2.2 Formation of social hierarchies 15

2.2.3 Treatment with SAFit2 16

2.2.4 Measurement of plasma cortisol 18

2.2.5 Measurement of relative transcript abundance by real-time RT-PCR 18

2.2.6 Statistical analysis 20

2.3 Results 21

2.4 Discussion 24

Appendix 2.1 58

Chapter 3: Stress responses and *fkbp5* transcript abundance in zebrafish with and without functional glucocorticoid receptors **60**

3.1 Introduction 61

3.2 Materials and methods 64

3.2.1 Experimental animals 64

3.2.2 Experiment 1. Developmental time course 66

3.2.3 Experiment 2. Exposure of adult and larval zebrafish to an acute stressor 66

3.2.4 Experiment 3. Exposure of adult zebrafish to chronic stressors 67

3.2.5 Tissue collection for adult zebrafish 68

3.2.6 Measurement of cortisol 69

3.2.7 Measurement of transcript abundance by real-time RT-PCR 69

3.2.8 Development of an *fkbp5*-KO line 70

3.2.9 Statistical analysis 71

3.3 Results 72

3.4 Discussion 73

References 86

List of Tables and Figures

Figure 1.1: Stress activates the HPI axis.....4

Figure 1.2: (A) In its inactive state, the GR is bound by several co-chaperones, including HSP90 and P23. **(B)** Once bound by cortisol, the GR exchanges FKBP5 for FKBP4 and the complex enters the nucleus where it binds to GREs to regulate the expression of target genes, including *fkbp5*. **(C)** In a cortisol-activated cell, the resulting FKBP5 can bind to HSP90 and GR, serving as a co-chaperone of GR, and keeping it in an inactive state where cortisol is unable to bind and the complex cannot translocate to the nucleus.....7

Figure 2.1: Behaviour scores **(A)** for dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of social interaction, or 4 d of interaction followed by 1 d of recovery with the divider replaced in the tank between the two fish. Plasma cortisol concentrations **(B)** in sham, dominant, and subordinate rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of social interaction, or 4 d of interaction followed by 1 d of recovery. 34

Figure 2.2: Transcript abundance of *fkbp5A* **(A)** and *fkbp5B* **(B)** in the POA of sham, dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of interaction, or 4 d of interaction followed by 1 d of recovery with the divider replaced in the tank between the two fish.....36

Figure 2.3: Transcript abundance of *fkbp5A* **(A)** and *fkbp5B* **(B)** in the head kidney in sham, dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of interaction, or 4 d of interaction followed by 1 d of recovery with the divider replaced in the tank between the two fish 38

Figure 2.4: Transcript abundance of *klf9* in the POA in sham, dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of interaction, or 4 d of interaction followed by 1 d of recovery with the divider replaced in the tank between the two fish 40

Figure 2.5: Transcript abundance of *fkbp4* in the POA **(A)** and head kidney **(B)** in sham, dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of interaction, or 4 d of interaction followed by 1 d of recovery with the divider replaced in the tank between the two fish. 41

Figure 2.6: Transcript abundance of *hsp90* in the POA **(A)** and head kidney **(B)** in sham, dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of interaction, or 4 d of interaction followed by 1 d of recovery with the divider replaced in the tank between the two fish 43

Figure 2.7: Transcript abundance of *gr1* **(A)**, *gr2* **(B)**, and *mr* **(C)** in the POA of sham, dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of interaction, or 4 d of interaction followed by 1 d of recovery with the divider replaced in the tank between the two fish 45

Figure 2.8: Transcript abundance of *gr1* **(A)**, *gr2* **(B)**, and *mr* **(C)** in the head kidney in sham, dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of interaction, or 4 d of interaction followed by 1 d of recovery with the divider replaced in the tank between the two fish 47

Figure 2.9: Transcript abundance of *fkbp5A* **(A)**, *fkbp4* **(B)**, and *hsp90* **(C)** in the liver of sham, dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of interaction, or 4 d of interaction followed by 1 d of recovery with the divider replaced in the tank between the two fish 49

Figure 2.10: Transcript abundance of *gr1* **(A)**, *gr2* **(B)**, and *mr* **(C)** in the liver of sham, dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of interaction, or 4 d of interaction followed by 1 d of recovery with the divider replaced in the tank between the two fish 51

Figure 2.11: Behaviour scores **(A)** and plasma cortisol concentrations **(B)** for dominant (dom) and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*), where subordinates were injected with vehicle or SAFit2 after 2 d of a 4 d social interaction period. 53

Table 2.1: Fish length and mass for rainbow trout used in all experiments.....55

Table 2.2: Gene-specific primers used for real-time RT-PCR.....56

Table 2.3: Summary of transcript abundance changes in subordinate rainbow trout compared to sham and/or dominant fish.....57

Figure A1: Distribution of *fkbp5A* **(A)** and *fkbp5B* **(B)** across tissues in rainbow trout (*Oncorhynchus mykiss*).....58

Figure A2: Distribution of *fkbp4* **(A)** and *β-actin* **(B)** across tissues in rainbow trout (*Oncorhynchus mykiss*).....59

Figure 3.1: SnapGene representation of the *fkbp5* gene deletion through CRISPR/Cas9, showing the target sites (T1-T4) for sgRNA and the binding sites for the screening primers (labelled *fkbp5*F1, F2, R1, R2) **(A),** as well as a schematic of the expected location of bands on an agarose gel for heterozygous and knockout fish based on screening primers **(B)**.....77

Figure 3.2: Transcript abundance of *fkbp5* in mWT or GR-KO larvae **(A)** and whole brain of adult male, mWT or GR-KO, zebrafish (*Danio rerio*) **(B)** under unstressed (baseline) conditions 78

Figure 3.3: Transcript abundance of *fkbp4* in mWT or GR-KO larvae **(A)** and whole brain of adult male, mWT or GR-KO, zebrafish (*Danio rerio*) **(B)** under unstressed (baseline) conditions 79

Figure 3.4: Transcript abundance of *hsp90* in mWT or GR-KO larvae **(A)** and whole brain of adult male, mWT or GR-KO, zebrafish (*Danio rerio*) **(B)** under unstressed (baseline) conditions 80

Figure 3.5: Plasma cortisol concentrations **(A)** and whole brain transcript abundance of *fkbp5* **(B)** in mWT or GR-KO male zebrafish (*Danio rerio*) prior to or after being subjected to a netting/air exposure stressor 81

Figure 3.6: Plasma cortisol concentrations in WT male zebrafish (*Danio rerio*) exposed to potential chronic stressors (hypoxia, low or high water temperature, or subordinate social status) for 4 d, together with values for corresponding control (con) fish exposed to normoxic or 28°C water, or of dominant social status.....82

Figure 3.7: 1.5% agarose gel of caudal fin-clipped *fkbp5*$^{-/-}$ F_1 zebrafish (*Danio rerio*) after PCR with F1/R1 primers **(A)**, and 1.5% agarose gel of caudal fin-clipped *fkbp5*$^{-/-}$ F_1 zebrafish after PCR with F2/R2 primers **(B)** 83

Table 3.1: Fish length and mass for zebrafish used in all experiments.....84

Table 3.2: Gene-specific primers used for real-time RT-PCR.....84

Table 3.3: Primers used for PCR assessment of fish genotype.....85

List of abbreviations

11βHSD1	11β-hydroxysteroid dehydrogenase type 1
11βHSD2	11β-hydroxysteroid dehydrogenase type 2
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
bp	Base pairs
CCAC	Canadian Council on Animal Care
cDNA	Complementary DNA
CRF	Corticotropin releasing factor
CRH	Corticotropin releasing hormone
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DNA	Deoxyribonucleic acid
dom	Dominant
dpf	Days post-fertilization
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme-linked immunoassay
FKBP4	FK506-binding protein 52
FKBP5	FK506-binding protein 51
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
gRNA	Guide RNA
HG	Hind gut
HK	Head kidney

HPA	Hypothalamic-pituitary-adrenal
hpf	Hours post-fertilization
HPI	Hypothalamic-pituitary-interrenal
HSF1	Heat-shock factor 1
HSP70	Heat shock protein 70
HSP90	Heat shock protein 90
i.p.	Intraperitoneal
KO	Knockout
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
mWT	Matched wildtype
PCA	Principal components analysis
PCR	Polymerase chain reaction
PEG-400	Polyethylene glycol 400
POA	Preoptic area
PVN	Paraventricular nucleus
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse transcription PCR
SEM	Standard error of the mean
sgRNA	Single guide RNA
sub	Subordinate
WT	Wildtype

Chapter 1: General introduction

1.1 Thesis overview

In fishes, as in other vertebrates, environmental challenges activate a coordinated series of behavioural and physiological responses, collectively termed the stress response, in which the elevation of glucocorticoid hormones (cortisol in fishes) plays a key role (Gorissen and Flik, 2016). Prolonged elevation of cortisol in response to such stressors can negatively impact the health of the fish (Gilmour et al., 2025), yet the factors that sustain cortisol elevation during chronic stress remain unclear (Best and Gilmour, 2022; Best et al., 2023). Studies on mammals have investigated the role of FK506-binding protein 51 (FKBP5) in chronic cortisol elevation (Rein, 2016; Zannas et al., 2016). However, less is known about the role of FKBP5 in fishes. Chapter 2 of this thesis focuses on rainbow trout (*Oncorhynchus mykiss*), using social subordination in juveniles as a model for chronic stress to investigate a potential role for FKBP5 in prolonged cortisol elevation. Chapter 3 focuses on zebrafish (*Danio rerio*), investigating patterns of FKBP5 expression across development and following an acute stressor in fish lacking the glucocorticoid receptor (GR). These studies aim to test the over-arching hypothesis that FKBP5 is involved in cellular regulation of the cortisol response to stress in fishes.

1.2 The stress axis

When a stressor is perceived by a teleost fish, the hypothalamic-pituitary-interrenal (HPI) axis is activated, leading to increases in the synthesis of cortisol by the interrenal cells of the head kidney (Tokarz et al., 2013). Specifically, perception of the stressor causes the release of corticotropin releasing factor (CRF) from neurons in the preoptic area (POA) of the brain onto the corticotropes of the anterior pituitary (Gorissen and Flik, 2016). The corticotropes, in turn, release adrenocorticotropic hormone (ACTH) into the circulation, and ACTH activates cortisol

biosynthesis in the interrenal cells (Fig. 1.1) (Gorissen and Flik, 2016). Cortisol exerts its effects by binding to and activating glucocorticoid (GR) or mineralocorticoid (MR) receptors, which then act as transcription factors to regulate the expression of genes that contain a glucocorticoid response element (GRE) (Faught et al., 2016; Zannas et al., 2016).

Acute elevation of cortisol can benefit the animal, for example by mobilizing energy stores that the animal can use to cope with the challenge (Gorissen and Flik, 2016; Schreck and Tort, 2016; Tokarz et al., 2013). However, chronic elevation of cortisol increases mortality, promotes immunosuppression, inhibits brain cell proliferation, alters metabolism, and decreases growth (Faught and Schaaf, 2024; Filby et al., 2010; Gilmour et al., 2025; Sadoul and Vijayan, 2016; Tea et al., 2019). Although multiple systems exist to lower cortisol, including clearance and catabolism, and negative feedback, cortisol can remain elevated during exposure to chronic stressors (Best et al., 2023; Tokarz et al., 2013). The main route for cortisol breakdown and clearance is via the hepatobiliary system (Best et al., 2023). Cortisol is first converted to cortisone, a process catalyzed by the enzyme 11ß-hydroxysteroid dehydrogenase type 2 (11βHSD2), and cortisone is then converted into water-soluble metabolites for excretion (Tokarz et al., 2013). Unlike mammals, fishes lack the enzyme 11ß-hydroxysteroid dehydrogenase type 1 (11βHSD1), which catalyzes the conversion of cortisone to cortisol, preventing this method of cortisol regulation (Tsachaki et al., 2017). Best et al. (2023) found no evidence that cortisol catabolism was impaired by subordinate social status in rainbow trout, yet subordinate fish exhibited prolonged elevation of cortisol.

The GR is the main mediator of negative feedback regulation of the HPI axis [or hypothalamic-pituitary-adrenal (HPA) axis in tetrapods] (Eachus et al., 2023; Zannas et al., 2016). Cortisol enters a cell by diffusion across the cell membrane and binds to GR in the

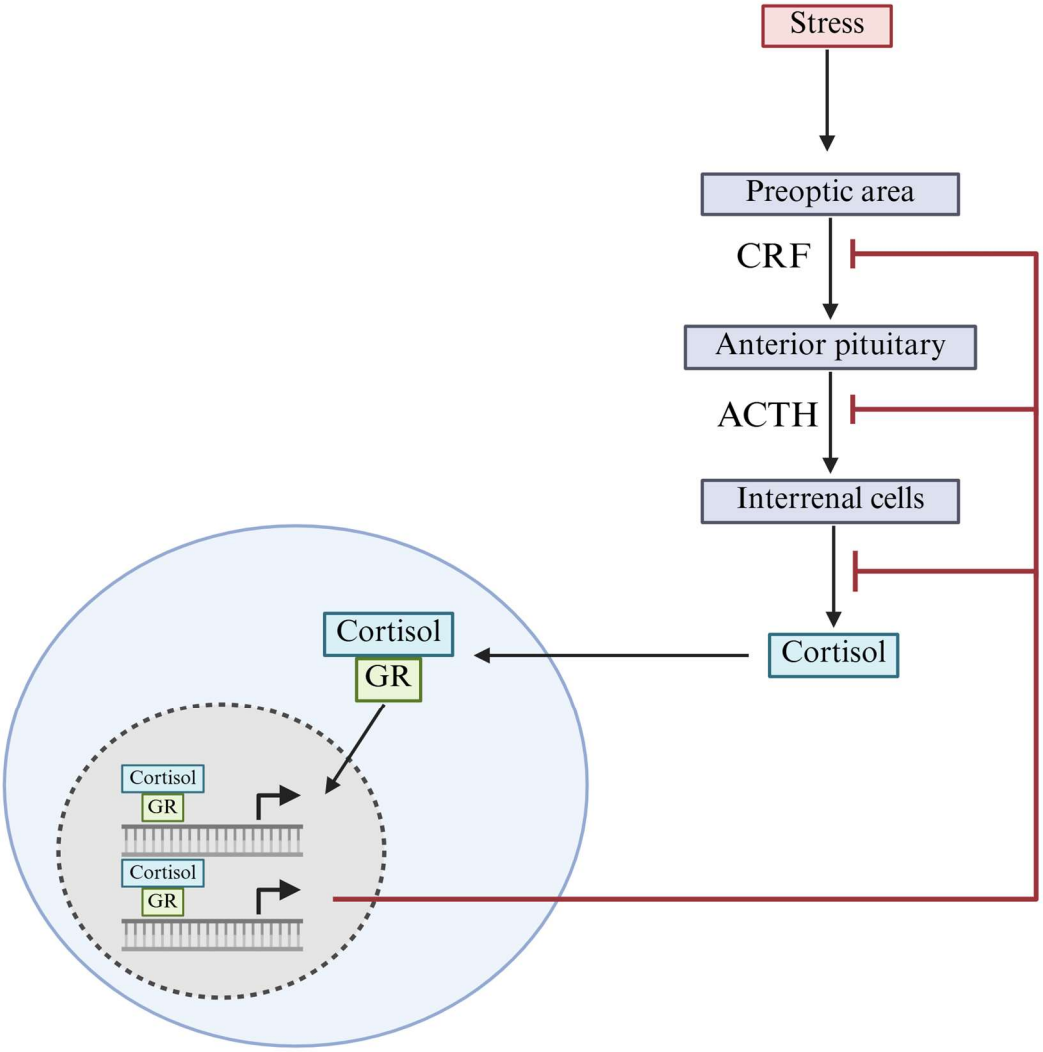


Figure 1.1: Stress activates the HPI axis. Neurons of the preoptic area of the brain release CRF onto the anterior pituitary, which stimulates the release of ACTH into the circulation. In turn, ACTH triggers the synthesis of cortisol in the interrenal cells of the head kidney. Cortisol enters a cell and binds to the glucocorticoid receptor (GR) in the cytoplasm. The cortisol-GR complex enters the nucleus and binds to glucocorticoid response elements (GRE) in the promoter regions of target genes, allowing transcriptional regulation of the target genes. When this regulation of gene expression occurs in the cells of the HPI axis, it leads to negative feedback inhibition of the HPI axis and a fall in circulating cortisol levels.

cytoplasm (Zannas et al., 2016). The GR-cortisol complex translocates to the nucleus where it acts as a transcription factor (Zannas et al., 2016), binding to GREs in the promoter region of target genes to regulate their expression (Eachus et al., 2023; Faught et al., 2016). The transcription of several genes that play key roles in stress axis activity is inhibited in this manner, ultimately resulting in the inhibition of the stress axis as a whole. For example, HPI axis inhibition occurs at the level of the POA to inhibit CRF signalling, and in the pituitary to inhibit ACTH release (Fryer and Peter, 1977; Fryer et al., 1984). In the head kidney, an ultra-short negative feedback loop is created by the actions of cortisol on the steroidogenic cells where it is produced (Bradford et al., 1992). Collectively, these actions create a negative feedback system that acts to rapidly attenuate cortisol production and lower circulating cortisol levels (Fig. 1.1) (Eachus et al., 2023). During chronic stress, however, negative feedback appears to be active, yet cortisol is sustained at elevated levels (Best et al. 2023). Best et al. (2023) suggested that subtle dysregulation of negative feedback, involving a change in the set-point at which cortisol levels are regulated, may account for this pattern. One possible mediator of such dysregulation of negative feedback is FKBP5.

1.3 GR and FKBP5

FKBP5 is a member of the FK506-binding protein family, which consist of immunophilins that are named for their ability to bind to the immunosuppressant drug FK506 (Rein, 2016; Zannas et al., 2016). FKBP5 possesses peptidyl-prolyl cis-trans isomerase activity, conferring on it the ability to act as a co-chaperone, that is, a molecule that changes the folding and activity of

a protein (Zannas et al., 2016). For example, FKBP5 serves as a co-chaperone of the GR (Zannas et al., 2016). While in an inactive state, GR is present in the cytoplasm bound to a heterocomplex consisting of heat shock protein 90 (HSP90), heat shock protein 70 (HSP70), p23, and the immunophilin FKBP5 (Faught et al., 2016). An important interaction of FKBP5 is with HSP90 – FKBP5 binds to HSP90 and together, they serve as co-chaperones of GR, to keep it in an inactive state (Faught et al., 2016; Rein, 2016; Zannas et al., 2016). Once cortisol binds to the GR, FKBP5 is exchanged for FK506-binding protein 52 (FKBP4), promoting translocation of the activated GR to the nucleus to initiate transcriptional regulation and allowing GR to mediate the effects of cortisol, including its negative feedback on the stress axis (Wochnik et al., 2005). Importantly, GR activation increases *fkbp5* transcription and translation, and the resulting increase in cellular FKBP5 abundance causes increased binding of FKBP5 to the GR (Zannas et al., 2016). *In vitro* studies, using mammalian cells, have reported that increased binding of FKBP5 to GR in this manner alters the sensitivity of GR, reduces its affinity for glucocorticoids, slows translocation of GR to the nucleus, and decreases transcriptional regulation by GR (Wochnik et al., 2005). Therefore, the induction of FKBP5 creates an ultra-short negative feedback loop (Fig. 1.2) on GR activity that, in the cells of the stress axis, has the capacity to impair negative feedback regulation (Zannas et al., 2016). This cellular negative feedback effect could result in the maintenance of high circulating cortisol levels. While cortisol also binds to MR, MR-FKBP5 interactions have rarely been studied. One study has shown that FKBP5 plays a role in the regulation of MR:GR balance in the hippocampus of rodents (Hartmann et al., 2021).

There is empirical support for effects of this cellular ultra-short negative feedback loop on whole-animal glucocorticoid levels. For example, treatment of stressed rodents with inhibitors of FKBP5, SAFit2 and benztropine, reduced circulating concentrations of corticosterone, the rodent

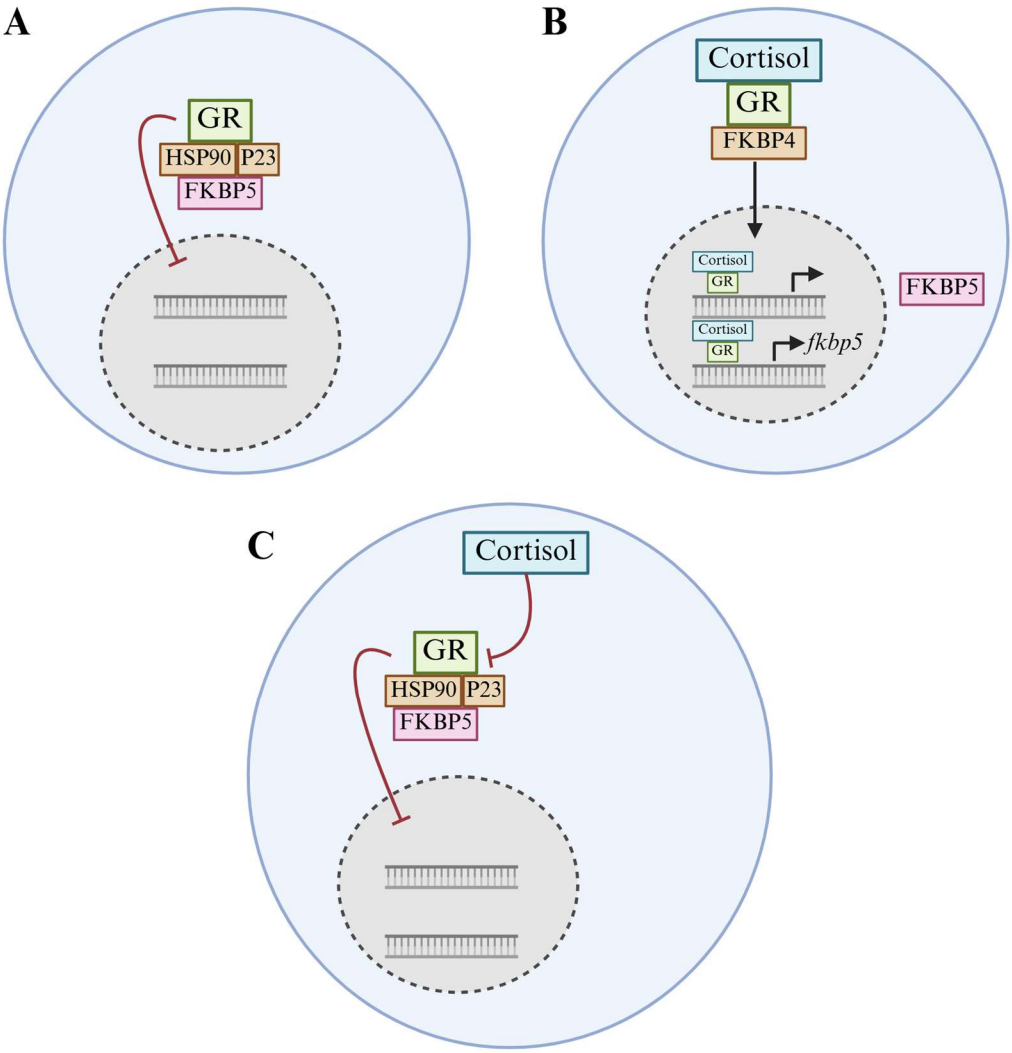


Figure 1.2: (A) In its inactive state, the GR is bound by several co-chaperones, including HSP90 and P23. **(B)** Once bound by cortisol, the GR exchanges FKBP5 for FKBP4 and the complex enters the nucleus where it binds to GREs to regulate the expression of target genes, including *fkbp5*. **(C)** In a cortisol-activated cell, the resulting FKBP5 can bind to HSP90 and GR, serving as a co-chaperone of GR, and keeping it in an inactive state where cortisol is unable to bind and the complex cannot translocate to the nucleus.

glucocorticoid stress hormone (Cruz et al., 2023). Treatment with SAFit2 also reduced glucocorticoid levels at the peak of their circadian rhythm (Gaali et al., 2015). Mice lacking functional expression of FKBP5 (*fkbp5*$^{-/-}$) exhibited lower basal corticosterone levels and decreased post-stress corticosterone levels relative to wildtype animals (Hartmann et al., 2012). Correspondingly, *fkbp5*$^{-/-}$ mice were considered to display high HPA flexibility (Zannas et al., 2016), which is defined as rapid, reversible changes in regulation of the HPA axis within an individual, in response to unexpected stressors (Zimmer et al., 2021). Because FKBP5 impairs negative feedback inhibition of the stress axis, it tends to decrease flexibility by preventing inactivation of the HPA axis after a stressor. For example, following exposure to stress, adult house sparrows (*Passer domesticus*) with lower *fkbp5* expression showed higher HPA flexibility, increasing their ability to cope with difficult conditions (Zimmer et al., 2021).

Whereas the role of FKBP5 in stress response regulation has received significant research attention in mammalian models, few studies have investigated whether FKBP5 plays a similar role in HPI axis regulation in fishes. The structure and function of the stress axis is highly conserved between the HPA axis in mammals and the HPI axis in fishes (Faught et al., 2016). Owing to the extra whole-genome duplication in fishes, fishes often have more paralogues of certain genes compared to mammals (Bury, 2017). Most fish have two paralogues of *gr*, for example, whereas zebrafish differ in having only one (Alsop and Vijayan, 2008; Bury, 2017). Similarly, searches using FEVER, an interactive web-based search engine for evolutionary transcriptomics in fishes, suggest that rainbow trout possess two paralogues of *fkbp5* whereas zebrafish possess only one (Montfort et al., 2024). Studies across several fish species, including zebrafish and Atlantic salmon (*Salmo salar*), have reported induction of *fkbp5* expression by GR activation (Chatzopoulou et al., 2017; Gans et al., 2021; Hartig et al., 2020; Tsoulia et al., 2025).

Hartig et al. (2020) reported that zebrafish embryos treated with cortisol showed elevated cortisol production and *fkbp5* expression in adulthood (Hartig et al., 2020). Additionally, *gr*^{s357} mutant zebrafish exhibited significantly lower *fkbp5* transcript abundance than wildtype fish (Eachus et al., 2023). Eachus et al. (2023) also analysed the *fkbp5* promoter region in zebrafish and found that it contained three GREs. However, beyond these observations, little is known about the regulation of *fkbp5* in relation to stress axis activity in fishes. Thus, the goal of the present thesis was to address this knowledge gap.

1.4 Hypotheses and objectives

The over-arching hypothesis of the present thesis is that FKBP5 is involved in cellular regulation of the stress response in fishes as in mammals. Based on this hypothesis, fish exposed to chronic stress would be predicted to exhibit elevated cortisol in conjunction with elevated *fkbp5* transcript abundance, and decreased GR activity. In Chapter 2, this hypothesis was investigated in rainbow trout, using dominant-subordinate social hierarchies as a model for chronic stress (Gilmour et al. 2025). Rainbow trout serve as a suitable study species for this work because subordinate fish have been shown to experience prolonged elevation of cortisol, and stress axis activity in subordinate fish has been examined in some detail (Best and Gilmour, 2022; Best et al., 2023; Best et al., 2024; Culbert and Gilmour, 2016; Gilmour et al., 2025; Jeffrey et al., 2012; Jeffrey et al., 2014). In Chapter 3, responses of *fkbp5* to acute and chronic stressors were investigated in developing and adult zebrafish. Further, the generation of a mutant zebrafish line lacking functional expression of FKBP5 was initiated using CRISPR/Cas9 gene-editing approaches. The zebrafish was used as a study species because of its rapid development and short generation time, the lack of a second GR, and the accessibility of this species to gene-

editing technology, which collectively provided the ability to both knock out *fkbp5* and use an already-established GR-knockout (GR-KO, *nr3c1*$^{-/-}$) line (Hong, 2024).

Chapter 2: A role for FKBP5 in modulating cortisol levels in subordinate rainbow trout

2.1 Introduction

In group-living fishes, limited access to resources can result in the development of social hierarchies (Gilmour et al., 2025; Milewski et al., 2022; Winberg and Sneddon, 2022). For example, when juvenile rainbow trout are held in pairs, dominant fish are aggressive toward their subordinate tank-mate and monopolize food and territory (Gilmour et al., 2025). As a result, the subordinates in these pairs experience chronic stress, as evidenced by the sustained elevation of cortisol (Best and Gilmour, 2022; Best et al., 2023; Gilmour et al., 2025). Cortisol can remain elevated for at least seven days in subordinate fish experiencing social interactions (Sloman et al., 2001), but fully recovers to baseline once the subordinate has been separated from the dominant for one to two days (Best and Gilmour, 2022; Culbert and Gilmour, 2016). As a result of this prolonged elevation of cortisol, subordinates experience negative physiological effects such as a switch in metabolism from an anabolic to a catabolic state, decreased growth and immune responses, as well as an attenuated cortisol response to an acute stressor (Jeffrey et al., 2014; Mennigen et al., 2022; Pickering and Pottinger, 1989).

The factors that mediate the sustained elevation of cortisol in subordinate trout remain unclear. Circulating cortisol levels reflect the opposing effects of cortisol synthesis in the interrenal cells of the head kidney and cortisol clearance and catabolism. Subordinate rainbow trout exhibit increased basal rates of cortisol production in the head kidney, a reflection at least in part of increased transcript abundance of the proteins responsible for the rate-limiting steps of steroidogenesis, namely steroidogenic acute regulatory protein and cytochrome P450 side chain cleavage enzyme (Best and Gilmour, 2022). Cortisol clearance appears to remain functional in subordinate trout, based on a cortisol challenge test as well as tissue rates of cortisol uptake and liver protein abundance of the cortisol inactivating enzyme 11ßHSD2 (Best et al., 2023). These

observations suggest that the maintenance of elevated cortisol is not caused by reductions in cortisol clearance or catabolism. A key regulator of circulating cortisol is negative feedback, which operates at all levels of the HPI axis (Bradford et al., 1992; Fryer and Peter, 1977; Fryer et al., 1984; reviewed by Bernier et al. 2009). Negative feedback relies on activation of the GR by cortisol, with the activated GR serving as a transcription factor to regulate (i.e. inhibit) the expression of key genes of the HPI axis (Eachus et al., 2023; Faught et al., 2016). Although Best et al. (2023) found that the key elements of negative feedback regulation of the HPI axis were present in subordinate trout as in dominant trout, subtle differences suggested that the set-point for negative feedback regulation was altered in subordinate trout, resulting in the regulation of cortisol at a new, elevated level (Best et al., 2023).

Studies in mammalian models have implicated FKBP5, a co-chaperone of the GR that acts as a negative regulator of GR activity and translocation, in dysregulation of the HPA axis during chronic stress (Hartmann, 2025; Zannas et al., 2016). For example, mice lacking functional expression of FKBP5 (*fkbp5*$^{-/-}$ mice) were less vulnerable to chronic social defeat stress, exhibiting among other effects lower corticosterone levels (Hartmann et al., 2012). Additionally, chronic administration of the FKBP5 inhibitor SAFit2, while mice were chronically stressed by social defeat and overcrowding, prevented the development of stress-induced behaviours and social withdrawal, suggesting that FKBP5 inhibition played a role in increasing resilience to chronic stress (Codagnone et al., 2022). Treatment with SAFit2 also reduced corticosterone levels at the peak of the circadian glucocorticoid rhythm in mice (Gaali et al., 2015). Notably, *fkbp5* transcript abundance increases in response to GR activation, setting up an ultra-short negative feedback loop in which a rise in cortisol increases transcription of *fkbp5*, with the resultant FKBP5 protein binding to GR and inhibiting its activity (Rein, 2016; Zannas et al.,

2016). In the tissues of the HPI axis, inactivation of the GR would, in turn, impair negative feedback regulation of glucocorticoid levels.

The present chapter examined the hypothesis that the prolonged elevation of cortisol in subordinate rainbow trout is mediated by an increase in *fkbp5* expression. I predicted that transcript abundance of *fkbp5* would be elevated in subordinate trout compared to their dominant counterparts as well as to sham-treated trout, which were fish that were handled as were the paired fish but housed alone. I examined pairs that interacted for 6 h, 4 d, or 4 d followed by 1 d of recovery, achieved by separating the members of a pair. These times were chosen to allow transcript abundance of *fkbp5* to be measured following the acute stressor of hierarchy formation, during the chronic stress of social subordination, or during recovery from a chronic stressor, respectively. I predicted that *fkbp5* transcript abundance would be elevated at 6 h of social interaction but GR activity would not be inhibited. At 4 d of social interaction, high *fkbp5* transcript abundance and inhibition of GR were both predicted to occur. A return of both *fkbp5* expression and GR activity to baseline levels was predicted to occur by 1 d of recovery. Expression of the cortisol-sensitive gene *klf9* was used as a read-out of GR activity. The transcript abundance of other key genes, including *hsp90*, *fkbp4*, *gr1*, *gr2*, and *mr* were also measured to provide a broader picture of the cellular players key to mediating cortisol's effects. These measurements were carried out on the POA, the site of initial activation of the stress response, and in head kidney tissue, which contains the interrenal cells where cortisol is synthesized (Gorissen and Flik, 2016). Liver tissue was also analyzed, to assess whether responses differed between tissues of the HPI axis (POA, head kidney) and non-HPI axis tissue (liver). Finally, we predicted that treatment of subordinate rainbow trout with the FKBP5 inhibitor, SAFit2, would alleviate the chronic elevation of cortisol.

2.2 Materials and methods

2.2.1 Experimental animals

Juvenile rainbow trout (mass 99.5 ± 5.9 g, n = 84; mean ± SEM) were obtained from Izumi Aquaculture (Campbellcroft, ON). Fish were held at the University of Ottawa in 1,275 L fibreglass tanks with a supply of 13°C aerated, flowing, dechloraminated city of Ottawa tap water ("system" water), under a 12:12 h light:dark photoperiod. Fish were fed 0.5% body mass daily by scattering commercial trout pellets across the water's surface, an approach that served to decrease hierarchy formation. The fish were acclimated to these conditions for at least 2 weeks before experimentation. All experimental protocols were approved by an institutional animal care committee (protocol BL-3668, University of Ottawa) and complied with the guidelines of the Canadian Council on Animal Care (CCAC) for the use of animals in research and teaching.

2.2.2 Formation of social hierarchies

Social hierarchies were formed using well-established protocols (Best et al., 2023; Culbert and Gilmour, 2016; Jeffrey et al., 2014). Fish were lightly anesthetized (0.05 g L^{-1} benzocaine; Sigma-Aldrich, Oakville, ON, CA) to measure fork length and mass, and to assess fin damage and identifying characteristics. Fish that were paired together were matched for size (length differences between the members of a pair averaged 0.3 ± 0.1 cm or 1.7 ± 0.2%; mass differences averaged 2.7 ± 0.4 g or 3.8 ± 0.5%, n = 20 pairs; see Table 2.1). Each pair was transferred to a 40 L tank that was supplied with flowing system water, and the members of the pair were separated by an opaque, perforated divider for an overnight recovery period (day 0). The next morning, the divider was removed and the fish were allowed to interact (day 1). Sham fish were handled in the same way but remained alone in their tank. Twice a day, in the morning (between 9 h and 11 h) and afternoon (13-15 h), the behaviour of each fish was scored during a 5

min observation period. Scores were based on position in the tank, number of aggressive acts, and feeding order when a single food pellet was added to the tank. Behaviours indicative of dominance include patrolling the tank, being aggressive towards the tankmate, and being first to feed, and the scoring scheme reflected these patterns (Best et al., 2023; Culbert and Gilmour, 2016; Jeffrey et al., 2014). Following the second observation period each day, pairs of fish were fed their usual food ration (0.5% body mass) without separating the members of the pair.

Three interaction periods were used, 6 h of interaction, 4 d of interaction, or 4 d of interaction followed by 1 d of recovery during which the divider was replaced to separate the members of the pair. At the end of the interaction and/or recovery period, the members of a pair were euthanized at the same time by immersion in a solution of benzocaine (0.5 g L^{-1}; Sigma-Aldrich), and mass, length and fin damage were assessed again. Blood samples were collected from the caudal vasculature with a 23 G needle and syringe coated with 0.5 mol L^{-1} ethylenediaminetetraacetic acid (EDTA). The blood samples were centrifuged at 11 000 *g* for 2 min, and the resulting plasma was collected, flash frozen in lN_2 and stored at -80°C for later analysis of cortisol concentrations. In addition, POA, head kidney, and liver tissue samples were collected from dominant and subordinate fish, flash frozen in lN_2 and stored at -80°C for later analysis of transcript abundance.

The mean scores for each behaviour over all observation periods were analysed by principal components analysis (PCA), to yield an overall behaviour score (PC1) for each fish. Within a pair, the fish with the higher behaviour score was assigned dominant status (Culbert and Gilmour, 2016; Jeffrey et al., 2014).

2.2.3 Treatment with SAFit2

SAFit2 ((2 S)-1-[(2 S)-2-cyclohexyl-2-(3,4,5-trimethoxyphenyl)acetyl]-2-piperidinecarboxylic acid, (1 R)-3-(3,4-dimethoxyphenyl)-1-[3-[2-(4-morpholinyl)ethoxy]phenyl]propyl ester; HY-102080-25MG; Cedarlane, Burlington, ON, CA) is a highly selective inhibitor of FKBP5. It was identified by means of an induced fit mechanism, and has been used in a variety of studies on mammalian cells and models (Codagnone et al., 2022; Gaali et al., 2015; Hartmann et al., 2015). The dose and timing of SAFit2 treatment were chosen based on published work, taking into account the need for the drug to have enough time to exert an effect and considering the difference in body temperature between mammals (37°C) and rainbow trout (13°C) (Codagnone et al., 2022; Gaali et al., 2015; Hartmann et al., 2015). Additionally, because only subordinate fish were treated with SAFit2, a period of interaction was required to assign social status before treatment. Size-matched rainbow trout were set up in pairs, as described above (length and mass differences between the members of a pair averaged 0.38 ± 0.07 cm and 4.9 ± 1.1 g, respectively, $N = 12$ pairs; see Table 2.1). Following the afternoon observation period on the second day of interaction (i.e. after ~48 h of interaction), social status was assigned based on behaviour scores. The members of a pair were separated by replacing the divider in the tank between them, and the subordinate fish was lightly anesthetized (0.05 g L^{-1} benzocaine; Sigma-Aldrich) and given an intraperitoneal (i.p.) injection of either SAFit2 (20 mg kg^{-1}) in saline containing 4% (v/v) ethanol, 5% (v/v) PEG-400 (Sigma-Aldrich) and 5% (v/v) Tween 80 (Sigma-Aldrich), or the vehicle alone (1.5 mL injection volume in both cases). The subordinate fish was replaced in its half of the tank and once it had returned to normal movement, the divider was removed to allow the members of the pair to continue to interact. At the end of the full 4 d interaction period, blood was collected as described above and plasma was flash frozen for later determination of plasma cortisol concentrations. Behaviour scores for the

entire 4 d observation period were calculated. Importantly, all fish identified as subordinate after 2 d of interaction were also assigned subordinate status at the end of the 4 d interaction period.

2.2.4 Measurement of plasma cortisol

Plasma cortisol levels were measured using a commercially available radioimmunoassay kit (MP Biomedicals, Solon, OH, USA) that has been validated for use in rainbow trout (Gamperl et al., 1994). The detection limit for this kit is 0.17 pg dL^{-1}, and assays were carried out as suggested by the manufacturer. In our hands, the inter- and intra-assay coefficients of variation average 7.6% and 5.6%, respectively.

2.2.5 Measurement of relative transcript abundance by real-time RT-PCR

Head kidney RNA was extracted by homogenizing the entire tissue sample from frozen using a handheld Kimble Pellet Pestle tissue grinder (Thermo Fisher Scientific, Waltham, MA, USA) in ice-cold TRIzol reagent (Invitrogen, Burlington, ON, CA). Total RNA was extracted from the whole POA or approximately 50 mg of liver tissue by sonicating with the Fisher Model 120 sonicator (Thermo Fisher Scientific) on ice in TRIzol reagent (Invitrogen), according to the manufacturer's instructions. The resultant RNA was suspended in nuclease-free water and a Nanodrop 2000c UV-Vis Spectrophotometer (Thermo Fisher Scientific) was used to quantify the RNA. Genomic DNA removal and the synthesis of cDNA were performed using 1 µg of total RNA with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Real-time RT-PCR was carried out using the Rotor-Gene SYBR Green PCR Kit (Qiagen) and a Rotor-Gene Q real-time PCR machine (Qiagen), following the manufacturer's instructions. Primers were identified from the literature or designed using Primer-BLAST (Table 2.2).

Salmonid fishes, such as rainbow trout, often have multiple paralogues owing to the salmonid-specific whole gene duplication event (Berthelot et al., 2014). For example, rainbow trout are known to have two paralogues of *gr* (Bury et al., 2003), and both were measured in the current study (*gr1* and *gr2*). Similarly, two paralogues were identified for *fkbp5* using FEVER and both were measured; for convenience, I labelled them *fkbp5A* (located on the seventeenth chromosome) and *fkbp5B* (located on the seventh chromosome). Only one paralogue was found for each of *fkbp4* and for *klf9*, while the primers for *hsp90* target the alpha subunit. Real-time RT-PCR reactions were carried out using a reaction volume of 10 µL, which consisted of 5 µL 2X SYBR mix, 0.2-0.5 µL forward primer (10 µM), 0.2-0.5 µL reverse primer (10 µM), 3-3.6 µL nuclease-free water, and 1 µL cDNA template. The PCR cycling conditions consisted of an initial 2-min activation at 95°C, followed by 40 cycles of 5-s denaturation at 95°C and 20-s annealing and extension at 60°C. Samples were assayed in duplicate, together with two no-template controls (which used H_2O instead of cDNA as template) and two no-RT controls (cDNA synthesis reaction carried out without reverse transcriptase). To confirm reaction efficiency, a standard curve was run for each primer set in each tissue (Table 2.2). Transcript abundance of the reference gene, *ß-actin*, was also measured in each tissue, and the stability of its expression across each social status/sampling time was confirmed. The relative fold-change of the target gene was normalized to the reference gene and expressed relative to the 6 h interaction sham group using the delta-delta Ct method (Pfaffl, 2001).

Prior to measuring relative transcript abundance by real-time RT-PCR, the tissue distributions of *fkbp5* and *fkbp4* transcript abundance were assessed. Transcripts were amplified by PCR with the PCR products being assessed on an agarose gel. A panel of tissues (POA, brain, eye, gill, heart, head kidney, stomach, hind gut, spleen, liver, muscle, and skin) was collected from each of

four rainbow trout that were euthanized (as described above) immediately upon netting from a holding tank. Total RNA was extracted with the Fisher Model 120 sonicator (Thermo Fisher Scientific) from whole POA, the remainder of the brain, and approximately 50 mg of the remaining tissues, as described above. Genomic DNA was removed with the DNase I, RNase free kit (Thermo Fisher Scientific). The synthesis of cDNA was performed on 1 µg of total RNA using the Applied Biosystems cDNA Reverse Transcription Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Amplicons were generated by PCR using BlasTaq 2X qPCR Mastermix (Applied Biological Materials, Richmond, BC, Canada) for the genes *fkbp5A*, *fkbp5B*, *fkbp4*, and *ß-actin* (for primers, see Table 2.2). The reaction volume was 10 µL, consisting of 5 µL BlasTaq mix, 0.5 µL forward primer (10 µM), 0.5 µL reverse primer (10 µM), 3 µL nuclease-free water, and 1 µL cDNA template. The PCR cycling conditions consisted of a 5-min activation at 95°C, followed by 40 cycles of a 15-s denaturation at 95°C, 15-s annealing at 60°C, 30-s extension at 72°C, then 5 min at 72°C. The PCR products were visualized on a 1.5% agarose gel stained with DyeNA-View Gel Stain (GeneBio Systems), together with a negative control and a GB-Ruler 100 bp DNA ladder (GeneBio Systems, Burlington, ON, CA) or Bio-Helix 50 bp DNA ladder (Froggabio, Concord, ON, Canada).

2.2.6 Statistical analysis

All statistical analyses were conducted using R-Studio (https://posit.co/download/rstudio-desktop/). Data are presented as means ± standard error of the mean (SEM). A Grubb's test was used to identify outliers. For cortisol concentrations and relative mRNA abundance of each gene, data were analysed by two-way analysis of variance (ANOVA) using interaction time and social status as factors. A Wilcoxon rank sum exact test was used to evaluate the impact of SAFit2 on cortisol levels, comparing subordinates for both treatments, as well as dominants and

subordinates within each treatment. Data were transformed where assumptions of normality and equal variance were not met, or an equivalent non-parametric test was used. Differences were considered to be statistically significant when $P < 0.05$.

2.3 Results

Across all interaction times, the pairs of rainbow trout formed strong social hierarchies based on behaviour scores that were positive in dominant fish and negative in subordinate fish (Fig. 2.1A). Plasma cortisol concentrations were significantly higher in subordinates than in dominant or sham fish independent of interaction period (Fig. 2.1B). In addition, a significant effect of interaction time was observed, with cortisol levels decreasing over time (Fig. 2.1B). The effect of time appeared to be driven at least in part by substantial changes in subordinate cortisol levels. To examine this trend, plasma cortisol levels were compared across social interaction treatments only within subordinate fish, revealing that cortisol was significantly higher after 6 h and 4 d of interaction than after 1 d of recovery (1-way ANOVA, $P_{time} = 0.005$).

There are two paralogues of *fkbp5* in rainbow trout, *fkbp5A* and *fkbp5B*, both with broad tissue expression. The expression of both paralogues was detected in each of the brain, POA, eye, gill, heart, head kidney, stomach, hind gut, spleen, liver, muscle, and skin (Figure A1). The distribution of *fkbp4* appeared to be somewhat less broad, with expression being detected in all tissues but the heart, spleen, and skin.

In the POA, there was no effect of social status on *fkbp5A* mRNA abundance, although a significant effect of time was observed (Fig. 2.2A). However, the transcript abundance of *fkbp5B* was approximately 60-fold higher in the POA of subordinates after 4 d of interaction compared to all other groups (Fig. 2.2B). In the head kidney, *fkbp5A* mRNA abundance was elevated in

subordinates relative to dominants after 4 d of interaction, and in both dominants and subordinates relative to sham fish after 6 h of interaction (Fig. 2.3A). Transcript abundance of *fkbp5B* was significantly elevated in subordinates independent of interaction time, and a significant effect of interaction time (independent of social status) was also observed (Fig. 2.3B).

The transcript abundance of *klf9* was measured as a potential read-out for GR activity. In the POA, it was elevated in subordinates compared to sham fish (Fig. 2.4). Transcript abundance of the major proteins with which FKBP5 interacts, including HSP90 and GR, were also assessed in the POA and head kidney, as was *fkbp4* mRNA abundance; FKBP4 replaces FKBP5 when the GR is activated by cortisol (Zannas et al., 2016). The mRNA abundance of *fkbp4* was elevated in the POA of dominant fish at 4 d of interaction compared to dominants after 1 d of recovery (Fig. 2.5A). In the head kidney, *fkbp4* transcript abundance was elevated in dominant fish relative to sham-treated fish after 6 h of interaction, and in dominant fish after 6 h of interaction relative to the 1 d recovery dominants (Fig. 2.5B). Transcript abundance of *hsp90* showed no significant effect of interaction time or status in the POA (Fig. 2.6A). In the head kidney, there was only an effect of time (Fig. 2.6B).

In the POA, transcript abundance of the two GR paralogues, *gr1* and *gr2*, as well as *mr*, were not affected by social status, but a significant effect of interaction time was observed, with all interaction periods differing from one another (Fig. 2.7). The mRNA abundance of *mr* was measured, because like the GR, the MR has been shown to be regulated by FKBP5 (Hartmann et al., 2021). In the head kidney, a significant effect of interaction time was observed for *gr1* and *gr2* mRNA abundance (Fig. 2.8A,B). However, subordinates showed an elevated transcript abundance of *mr* compared to shams in head kidney (Fig. 2.8C).

The mRNA abundance of each gene was also measured in the liver, a tissue that is not part of the HPI axis but that responds to cortisol, with the goal of determining whether responses differed between the HPI axis and other tissues. Transcript abundance of *fkbp5A* was elevated at 6 h of interaction in liver tissue (Fig. 2.9A). Although a trend for social status to impact *fkbp5A* transcript abundance was present, it did not reach statistical significance (Fig. 2.9A). Transcript abundance of *fkbp5B* was not measured in liver because the threshold for detection (C_q) was too high for reliable detection. Transcript abundance of *fkbp4* was significantly lower in subordinates after 4 d compared to all other groups (Fig. 2.9B). The transcript abundance of *hsp90* was significantly affected by interaction time, with all interaction periods differing from one another, and social status, with subordinates showing lower transcript abundance than sham-treated fish (Fig. 2.9C). The mRNA abundance of *gr1* and *gr2* were significantly affected by both interaction time and social status, with sham-treated fish exhibiting higher transcript abundance than subordinates (Fig. 2.10A,B). A significant effect of interaction time was also observed for *mr* transcript abundance, where all periods differed from each other (Fig. 2.10C). Additionally, a trend for social status affecting *mr* transcript abundance was observed, but statistical significance was not reached (Fig. 2.10C).

Treatment of subordinate trout with the FKBP5 inhibitor SAFit2 was used to evaluate whether inhibition of FKBP5 lowered cortisol levels in subordinate fish (only subordinate fish were treated with SAFit2 or the vehicle). The behaviour scores for these pairs showed that strong social hierarchies were formed regardless of whether subordinate fish were treated with vehicle or SAFit2 (Fig. 2.11A). Although a comparison of cortisol values between vehicle- and SAFit2-treated subordinates did not reveal a significant difference, vehicle-treated subordinates tended to have significantly higher cortisol values than the dominant fish with which they were paired,

whereas no significant difference in cortisol values was detected between dominant fish and SAFit2-treated subordinates (Fig. 2.11B).

2.4 Discussion

The present chapter aimed to determine whether the chronic cortisol elevation experienced by subordinate rainbow trout is mediated by FKBP5. As predicted, subordinate trout exhibited elevated plasma cortisol after 6 h and 4 d of social interaction. In the initial phases of social hierarchy formation, both dominants and subordinates are acutely stressed, with cortisol in dominants returning to baseline levels after a few hours (Øverli et al., 2000); reviewed by (Gilmour et al., 2025). By contrast, subordinates maintain the cortisol elevation for days or weeks, allowing the 4 d interaction period to represent a chronic stressor (Ejike and Schreck, 1980; Elofsson et al., 2000; Laidley and Leatherland, 1988; Pottinger and Pickering, 1992; Sloman et al., 2001; Winberg and Lepage, 1998). Once physically separated from the dominant, cortisol in subordinates returns to baseline levels after 1 to 2 d (Best et al., 2023; Culbert and Gilmour, 2016). Here, cortisol in most subordinates had recovered to baseline levels after 1 d, with two fish maintaining cortisol levels indicative of stressed fish.

In rainbow trout, there are two paralogues of *fkbp5,* termed *fkbp5A* and *fkbp5B* for the purposes of the present study. Both are widely expressed among tissues and are expected to possess cis-trans isomerase activity to alter protein folding. A search of the literature failed to reveal previous work on *fkbp5* in rainbow trout, and therefore the relative contribution of each paralogue to FKBP5 function remains unknown. In the present study, transcript abundance of the *fkbp5B* paralogue was 60-fold higher in the POA of subordinates after 4 d of interaction compared to all other groups. Notably, elevated expression of *fkbp5B* was present only in

subordinates, and only after 4 d of social interaction, a finding that is consistent with the prediction of elevated *fkbp5* expression in response to chronic stress. Similar results were reported in mice, where exposure to chronic mild stress increased the expression of *fkbp5* in the hippocampus and prefrontal cortex (Guidotti et al., 2013). The POA in fishes is the site of CRF synthesis; the release of CRF following perception of a stressor initiates HPI axis activity, ultimately increasing the synthesis of cortisol (Gorissen and Flik, 2016). Because of its role in initiating HPI axis activity, negative feedback inhibition of CRF synthesis and/or release takes on particular importance in regulating circulating cortisol levels. If increased expression of *fkbp5* in the POA of subordinate fish translates into higher FKBP5 abundance and FKBP5 binding to GR, resulting in GR inhibition, then negative feedback control of CRF synthesis and/or release could be impaired (Best et al., 2023; Zannas et al., 2016). Previous work suggests that continued stress axis activation contributes to subordinate cortisol elevation, as evidenced by elevated mRNA abundance of *crf* in the POA of subordinate fish in many although not all studies of rainbow trout pairs (Best et al., 2023; Best et al., 2024; Doyon et al., 2003; Jeffrey et al., 2012). By lowering negative feedback inhibition at the initial stage of the HPI axis, FKBP5 may be a key contributor to maintenance of stress axis activity (Best et al., 2023). In mice, for example, overexpression of *fkbp5* in the paraventricular nucleus (PVN) of the hypothalamus – the cite of corticotropin releasing hormone (CRH) synthesis in mammals – led to increased blood corticosterone levels, increased activation of the HPA axis, and a mimicking of a chronically stressed phenotype in other tissues (Häusl et al., 2021). Interestingly, POA transcript abundance of the paralogue *fkbp5A* was not affected by social status, suggesting different roles for the two *fkbp5* paralogues following exposure to stress.

In the head kidney, where cortisol is synthesized by interrenal cells, *fkbp5B* transcript abundance was elevated in subordinates independent of interaction period, although at 1 d of recovery, only one subordinate fish (of six) exhibited substantially elevated *fkbp5B* transcript abundance. Notably, this subordinate also exhibited the highest POA *fkbp5B* mRNA abundance among the recovering subordinates. This observation is consistent with the prediction of a direct association between elevated cortisol and elevated *fkbp5* abundance. Unlike in POA, there was also evidence of effects of social status on the transcript abundance of *fkbp5A* in head kidney tissue – at 4 d of social interaction, transcript abundance of *fkbp5A* was elevated in subordinates compared to dominants. In addition, *fkbp5A* abundance was elevated in both dominants and subordinates after 6 h of social interaction. The acute stress and accompanying elevation of cortisol experienced by both dominants and subordinates during establishment of social status (Øverli et al., 2000) could explain this result. By 4 d, on the other hand, it is likely that the continuing elevation of *fkbp5A* and *fkbp5B* contributes to the maintained elevation of cortisol in subordinate fish. Comparison of the patterns of *fkpb5* expression between POA and head kidney also suggests that the regulation of *fkbp5* expression in this social stress scenario may be tissue specific. Many studies have examined the role of FKBP5 in brain, but few have explored it in the adrenal glands, the mammalian homologue of the fish head kidney (Aman et al., 2025; Brix et al., 2022; Häusl et al., 2021; van Doeselaar et al., 2023). Interestingly, deletion of *fkbp5* specifically from the adrenal cortex of C57Bl/6 mice did not alter basal corticosterone levels, the corticosterone response to an acute stressor, or corticosterone levels following exposure to a chronic social defeat stress (Aman et al., 2025). These findings suggest that the role of FKBP5 in regulating circulating glucocorticoid levels primarily occurs at higher levels of the stress axis. However, because of the nature of the FKBP5-GR negative feedback loop, it remains difficult to

distinguish cause and effect, i.e. to determine whether and when cortisol-induced increases in *fkbp5* transcript abundance begin to contribute to the elevation of circulating cortisol.

The present study assessed transcript abundance of *klf9* as a potential read-out of GR activity. In a chronic stress scenario, increased binding of FKBP5 to GR would be expected to inhibit GR activity and hence transcriptional regulation of cortisol-sensitive genes (Wochnik et al., 2005). If FKBP5 is increased in subordinate fish, then GR activity would be predicted to be inhibited, leading to a relative decrease in *klf9* expression. However, transcript abundance of *klf9* was significantly higher in the POA of subordinate relative to dominant trout in the current study, with no significant effect of social interaction time. Despite the apparent absence of an effect of social interaction time, the highest *klf9* transcript abundance were observed in subordinate fish after 4 d of interaction, the time at which inhibition of GR activity was anticipated to occur. These results suggest that either inhibition of GR activity did not occur, or that *klf9* transcript abundance is not an appropriate read-out for GR activity. Although *klf9* expression is regulated by cortisol-mediated GR activity, it also appears to be involved in the regulation of *fkbp5* (Gans et al., 2020; Gans et al., 2021), suggesting that it may not be a suitable indicator of whether FKBP5 has inhibited GR activity. Thus, an alternative approach is needed to assess GR activity. Mammalian studies have instead used reporter assays in SK-N-MC, HEK, and HeLA cells, which have shown that FKBP5 inhibits GR-dependent transcription (Wochnik et al., 2005), and it is likely that a similar approach is needed for rainbow trout.

Although the findings must be considered preliminary given the small number of trout included in the trial, treatment of subordinate trout with the FKBP5 inhibitor SAFit2 appeared to ameliorate the chronic elevation of cortisol in these fish. That is, whereas cortisol levels in subordinates treated with vehicle tended to be higher than those in the dominants with which

they were paired after 4 d of social interaction, cortisol levels in subordinates treated with SAFit2 did not differ from those in their dominant tankmates. This finding is consistent with work in mice, where treatment with SAFit2 decreased peak corticosterone levels and prevented stress-induced behaviours (Codagnone et al., 2022; Gaali et al., 2015). However, no significant difference in cortisol was detected between vehicle- and SAFit2-treated subordinate trout, although the lower variance in values for SAFit2-treated fish suggested that a significant difference might emerge with a larger sample size. To my knowledge, the present study was the first use of SAFit2 in a fish, and it was assumed that the induced fit mechanism of SAFit2 to rodent FKBP5 would also function in trout FKBP5A and FKBP5B. An *in silico* analysis of SAFit2 interactions with FKBP5A and FKBP5B would be helpful in testing this assumption. The high cost of the drug coupled with the relatively large size of the fish available for the experiment were constraints on sample size. In addition, the SAFit2 dose and timing of treatment were extrapolated from rodent studies (Codagnone et al., 2022). The SAFit2 dose used in trout, 20 mg kg^{-1}, matched that found to be effective in whole-animal studies in mice and rats (Codagnone et al., 2022; Cruz et al., 2023). In these studies, the effectiveness of SAFit2 was also found to be sensitive to the timing of drug administration, where treatment produced an effect 16 h prior to testing, but not 1 h prior to testing (Codagnone et al., 2022; Hartmann et al., 2015). Here, the timing of drug administration was adjusted to reflect the large difference in body temperature between rodents (37°C) and trout (13°C). Again, however, the limited amount of SAFit2 available meant that only a single administration time could be tested. Additional experiments, potentially with a different timeline of drug administration, are required to confirm whether SAFit2 is effective in alleviating the chronic elevation of cortisol in subordinate trout.

To provide a broader picture of the cellular environment in which *fkbp5* is involved, I also measured the transcript abundance of *fkbp4*, *hsp90*, *gr*, and *mr* (Table 2.3). Upon glucocorticoid binding to the GR, FKBP4 replaces FKBP5, allowing the GR-cortisol complex to enter the nucleus (Zannas et al., 2016). In the POA and head kidney, *fkbp4* mRNA abundance was significantly higher in dominants after 4 d and 6 h of social interaction, respectively, compared to dominants after 1 d of recovery. These patterns may suggest higher FKBP4 abundance in dominant fish. Although dominant fish experience acute stress during hierarchy formation, negative feedback and cortisol clearance mechanisms operate effectively to return circulating cortisol to baseline levels within a few hours (Best et al., 2023). It is tempting to speculate that elevated *fkbp4* transcript abundance following the period of acute stress 'primes' cellular mechanisms so as to allow the fish to respond effectively to additional stressors. Similarly, Jeffrey et al. (2012) reported differences in transcript abundance of genes involved in cortisol synthesis between dominant and subordinate trout that suggested that the acute stress of hierarchy formation prepares the stress axis for subsequent responses, although these responses were not detected by Best & Gilmour (2022). Few studies have measured *fkbp4* abundance in relation to stress. In murine AtT-20 corticotropes (ACTH-producing cells), treatment with dexamethasone, a synthetic glucocorticoid drug, decreased *fkbp4* mRNA and protein abundance while increasing *fkbp5* abundance (Kageyama et al., 2021).

Although prior work identified significant effects of social status on HSP90 protein abundance in the brain of rainbow trout (Currie et al., 2010), no significant effects of social status on *hsp90* transcript abundance were detected in the POA or head kidney in the present study. This finding suggests that differences with social status are regulated primarily at the protein level. Because HSP90 plays many roles, several regulatory systems modulate its activity

(Prodromou, 2016). Heat-shock factor 1 (HSF1) is the major regulator of *hsp90* expression, and post-translational modifications are important for differentiating the role of HSP90 complexes (Prodromou, 2016).

Increased abundance of FKBP5 was not expected to alter transcript or protein abundance of GR, but rather to inhibit its activity. Nevertheless, transcript abundance of *gr1* and *gr2* were assessed for completeness. No effect of social status was detected for either *gr* paralogue in either POA or head kidney. A similar lack of effect of social status on *gr* transcript abundance was observed in the POA and pituitary of trout after 4 d of interaction, or 4 d of interaction followed by 1 d or 2 d of individual recovery (Best et al., 2023). However, protein abundance of GR after 2 d of recovery was decreased in subordinates (Best et al., 2023).

In the hippocampus of mice, the inhibition of MR results in decreased mRNA abundance of *fkbp5* (Hartmann et al., 2021). The MR is also involved in the perception of stress, the maintenance of cortisol homeostasis after acute stressors, and the regulation of key genes of the HPI axis (Faught and Vijayan, 2018; Zannas et al., 2016). Therefore, *mr* transcript abundance was assessed in the present study. An effect of social status was observed in the head kidney, but not the POA, with subordinates having higher *mr* mRNA abundance compared to sham fish. Similarly, Best et al. (2023) did not detect effects of social status on *mr* transcript abundance in the POA but reported lower *mr* abundance in the pituitary of subordinates relative to dominants. Notably, in the present study *fkbp5B* mRNA abundance was also significantly elevated in subordinate head kidney tissue and subordinates also tended to show elevated transcript abundance of *fkbp5A* at 6 h and 4 d of social interaction. However, more work is needed to determine whether and how MR and FKBP5 interact.

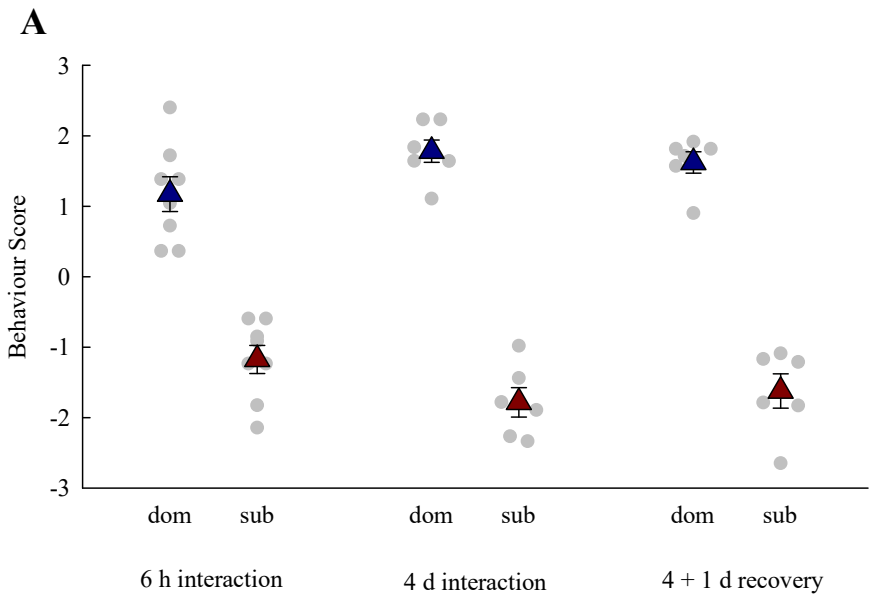
To assess the extent to which transcript abundance responses were specific to stress axis tissues, transcript abundance of the same panel of genes were measured in the liver with the exception of *fkbp5B*, where expression appeared to be too low to be reliably quantified by real-time RT-PCR (Table 2.3). For *fkbp5A*, no significant effects of social status were detected although five of seven subordinate fish showed substantial transcript abundance after 6 h of social interaction. In addition, transcript abundance of *fkbp4* was significantly lower in subordinates after 4 d of social interaction than in any other group. These responses appeared to be less marked than those observed in POA or head kidney. For both *hsp90* and *gr1* but not *gr2* or *mr*, sham fish exhibited higher transcript abundance than subordinates. Similarly, Jeffrey et al. (2012) observed lower transcript abundance of *gr1* and *gr2* and lower GR protein abundance in the liver of subordinate fish, which were interpreted as a protective mechanism to minimize liver GR stimulation by the persistent cortisol elevation caused by subordinate status. Collectively, these data suggest that *fkbp5* responses in the tissues of the HPI axis, i.e. POA and head kidney, differ from those in a cortisol-responsive target tissue (liver), likely as a result of regulation of HPI axis activity.

The findings of the present study suggest that FKBP5 plays a role in the maintained elevation of circulating cortisol in subordinate rainbow trout. However, several limitations preclude drawing a stronger conclusion. Firstly, transcript abundance were measured in POA and head kidney, both of which are heterogeneous tissues containing cell types beyond those of interest to the present study, i.e. CRF-producing neurons and cortisol-producing interrenal cells, respectively (Bernier et al., 2009; Gallo and Civinini, 2003; Hontela et al., 2008). Particularly for head kidney, where the cortisol-producing interrenal cells are estimated to account for only one in 8,000 cells (Hontela et al., 2008), transcript abundance responses measured at the tissue level

may not be accurate estimates of responses in the relevant cells. It is also worth noting that significant effects of interaction time that were independent of social status were observed on the transcript abundance of a number of genes. These effects were likely a consequence of running trials for different interaction periods separately. Since the effects were independent of social status, they were generally not taken into consideration in interpreting the data. Also, transcript abundance, rather than protein abundance were measured, and changes in mRNA abundance may not predict changes at the protein level (Liu et al., 2016). Although FKBP5 antibodies are available for use in rodents (Kubiak et al., 2025; Liu et al., 2023), antibodies specific to trout FKBP5A and FKBP5B are needed to establish whether FKBP5 protein abundance increases in subordinate trout. A key challenge for the present study was determining whether increases in *fkbp5* transcript abundance lead to inhibition of GR activity. Increased FKBP5 in HeLa and H4-II-E-C3 cells inhibited the activity of the GR target genes metallothionein (MT) and tyrosine aminotransferase (TAT) (Wochnik et al., 2005), and therefore a comparable approach – measurement of the transcript abundance of the GR-responsive gene *klf9* – was used in the present study. However, as discussed above, the results obtained with this approach were difficult to interpret, and therefore an alternative read-out of GR activity is needed. Inhibition of FKBP5 provides a whole-animal approach to assessing its role in the prolonged elevation of cortisol in subordinate trout, allowing the effect of cortisol on *fkbp5* transcript abundance to be distinguished from the effect of FKBP5 binding to GR. Despite the constraints present in the SAFit2 trial (see above), the results of this trial were consistent with the hypothesis that FKBP5 plays a role in the maintained elevation of cortisol in chronic stress.

In conclusion, a role for FKBP5 in maintaining elevated cortisol concentrations in subordinate trout could not be definitely demonstrated in the present study. However, the patterns

of *fkbp5* transcript abundance in POA and head kidney (Table 2.3) in conjunction with the apparent effect of FKBP5 inhibition on cortisol levels in subordinate relative to dominant trout after 4 d of social interaction supports a role for FKBP5 in contributing to the maintained elevation of cortisol in subordinate rainbow trout.


A
Behaviour Score
3
2
1
0
-1
-2
-3
dom
sub
dom
sub
dom
sub
6 h interaction
4 d interaction
4 + 1 d recovery

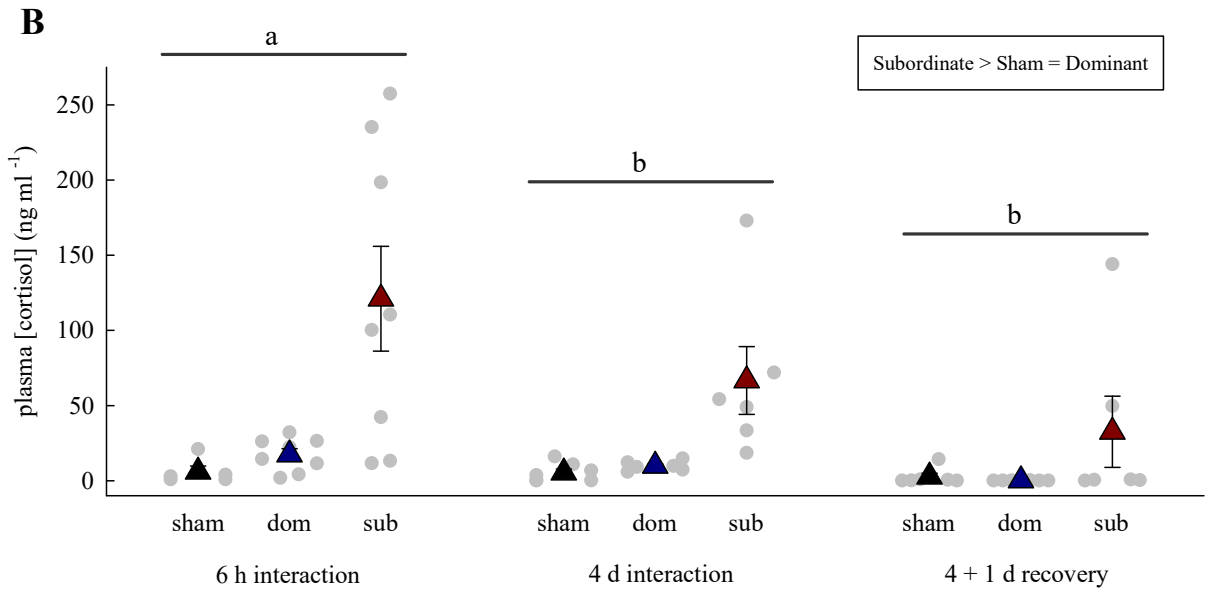

B
a
b
b
Subordinate > Sham = Dominant
plasma [cortisol] (ng ml -1)
250
200
150
100
50
0
sham
dom
sub
sham
dom
sub
sham
dom
sub
6 h interaction
4 d interaction
4 + 1 d recovery

Figure 2.1: Behaviour scores **(A)** for dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of social interaction, or 4 d of interaction followed by 1 d of recovery with the divider replaced in the tank between the two fish. Plasma cortisol concentrations **(B)** in sham, dominant, and subordinate rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of social interaction, or 4 d of interaction followed by 1 d of recovery. In both panels, data are presented as means ± SEM (n = 6-8 per group), with the grey circles representing values for individual fish. In panel B, interaction times that share a letter are not significantly different from one another, and significant effects of social status are noted on the figure (2-way ANOVA, $P_{time} < 0.001$, $P_{status} < 0.001$, $P_{time\ x\ status} = 0.21$).

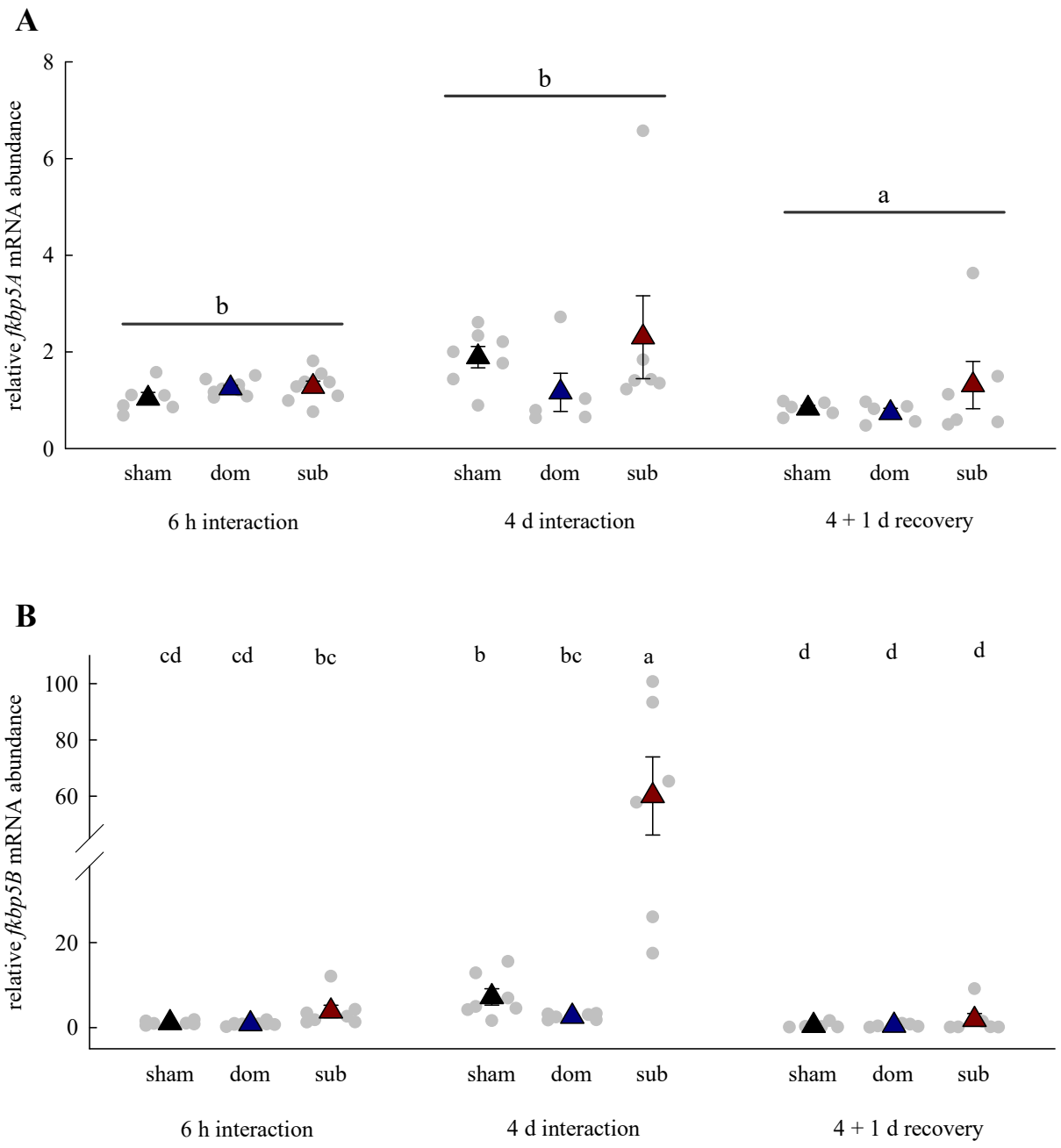
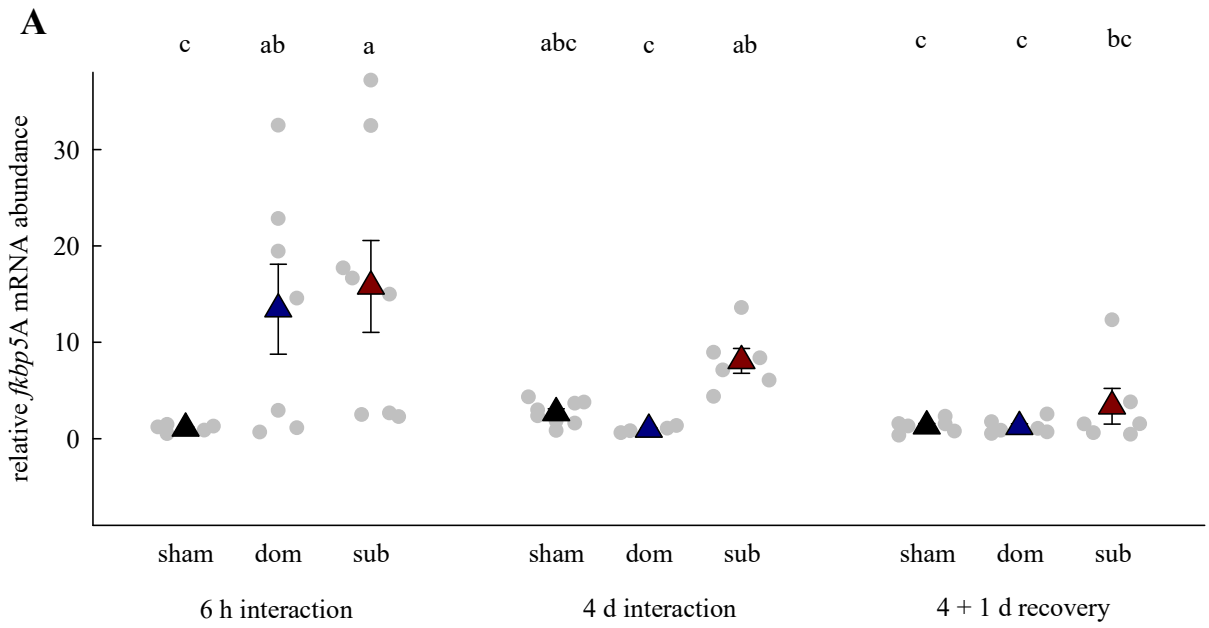

A
relative fkbp5A mRNA abundance
8
6
4
2
0
b
b
a
sham
dom
sub
6 h interaction
4 d interaction
4 + 1 d recovery
B
relative fkbp5B mRNA abundance
100
80
60
20
0
cd
cd
bc
b
bc
a
d
d
d
sham
dom
sub
6 h interaction
4 d interaction
4 + 1 d recovery

Figure 2.2: Transcript abundance of *fkbp5A* **(A)** and *fkbp5B* **(B)** in the POA of sham, dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of interaction, or 4 d of interaction followed by 1 d of recovery with the divider replaced in the tank between the two fish. Data are presented as means ± SEM (n = 5-8 per group), with the grey circles representing values for individual fish. In panel A, interaction times that share a letter are not significantly different from one another, whereas in panel B, groups that share a letter are not significantly different from one another (**A**: 2-way ANOVA on ranks, $P_{time} = 0.01$, $P_{status} = 0.47$, $P_{time\ x\ status} = 0.26$; **B**: 2-way ANOVA, $P_{time} < 0.001$, $P_{status} < 0.001$, $P_{time\ x\ status} = 0.01$).


A
c
ab
a
abc
c
ab
c
c
bc
relative fkbp5A mRNA abundance
30
20
10
0
sham
dom
sub
sham
dom
sub
sham
dom
sub
6 h interaction
4 d interaction
4 + 1 d recovery

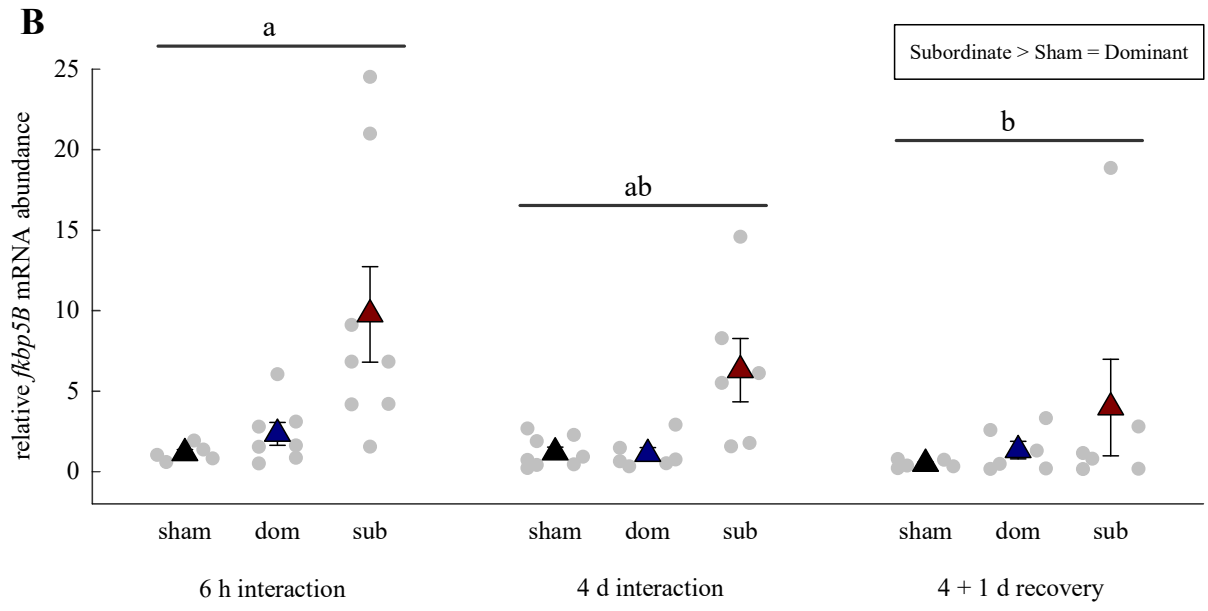

B
a
ab
b
Subordinate > Sham = Dominant
relative fkbp5B mRNA abundance
25
20
15
10
5
0
sham
dom
sub
sham
dom
sub
sham
dom
sub
6 h interaction
4 d interaction
4 + 1 d recovery

Figure 2.3: Transcript abundance of *fkbp5A* **(A)** and *fkbp5B* **(B)** in the head kidney in sham, dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of interaction, or 4 d of interaction followed by 1 d of recovery with the divider replaced in the tank between the two fish. Data are presented as means ± SEM (n = 6-8 per group with the exception of 4 d interaction doms, where n = 4), with the grey circles representing values for individual fish. In panel A, groups that share a letter are not significantly different from one another, whereas in panel B, interaction times that share a letter are not significantly different from one another, and significant effects of social status are noted on the figure (2-way ANOVA, **A**: $P_{time} < 0.001$, $P_{status} < 0.001$, $P_{time\ x\ status} < 0.001$; **B**: $P_{time} < 0.001$, $P_{status} < 0.001$, $P_{time\ x\ status} = 0.50$).

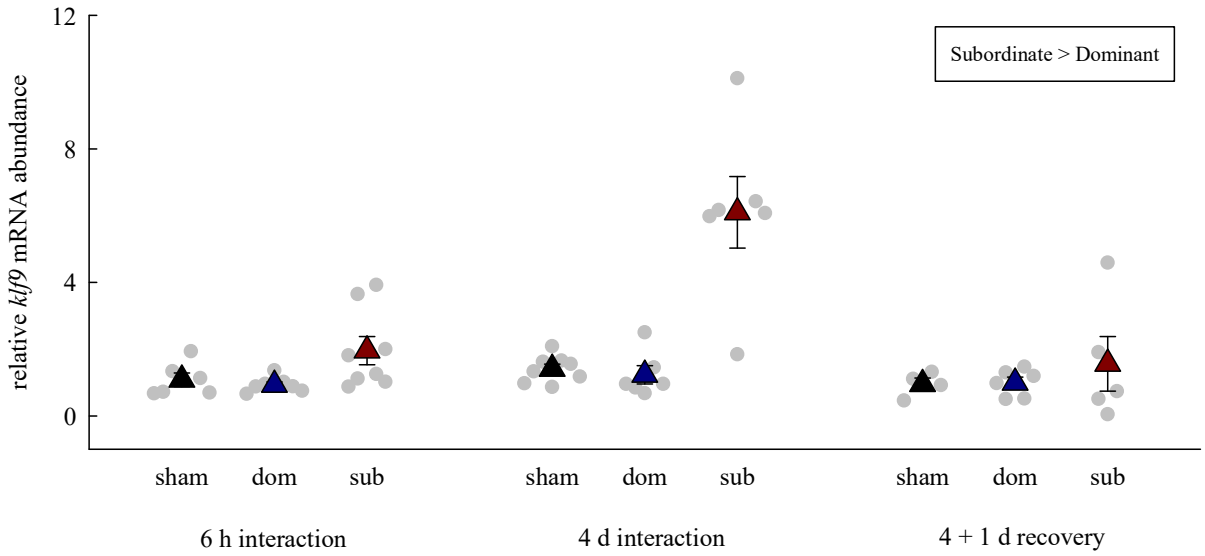


Figure 2.4: Transcript abundance of *klf9* in the POA of sham, dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of interaction, or 4 d of interaction followed by 1 d of recovery with the divider replaced in the tank between the two fish. Data are presented as means ± SEM (n = 5-8 per group with the exception of 1 d recovery shams, where n = 4), with the grey circles representing values for individual fish. Significant effects of social status are noted on the figure (2-way ANOVA on ranks, $P_{time} = 0.13$, $P_{status} = 0.01$, $P_{time \times status} = 0.32$).

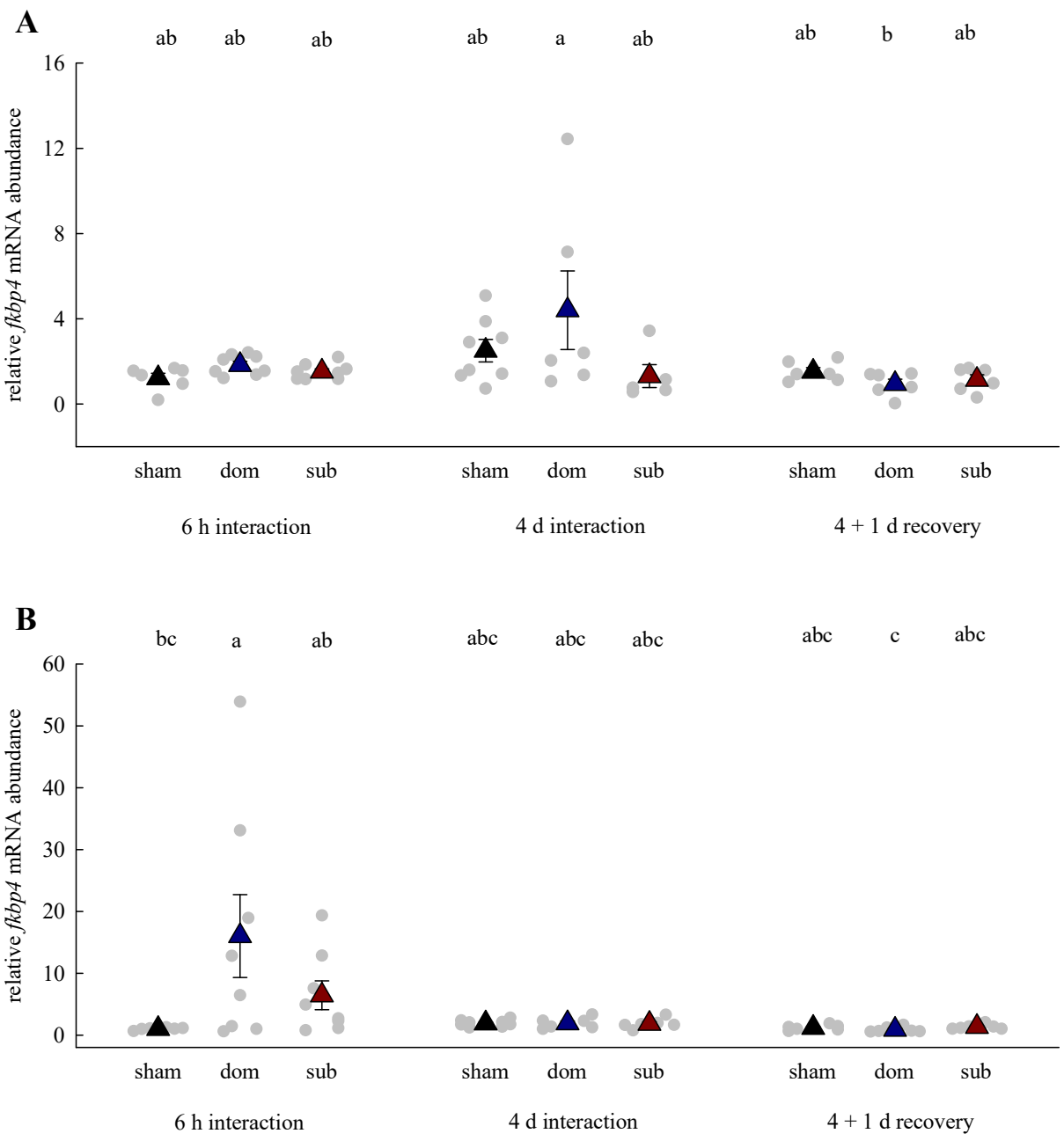
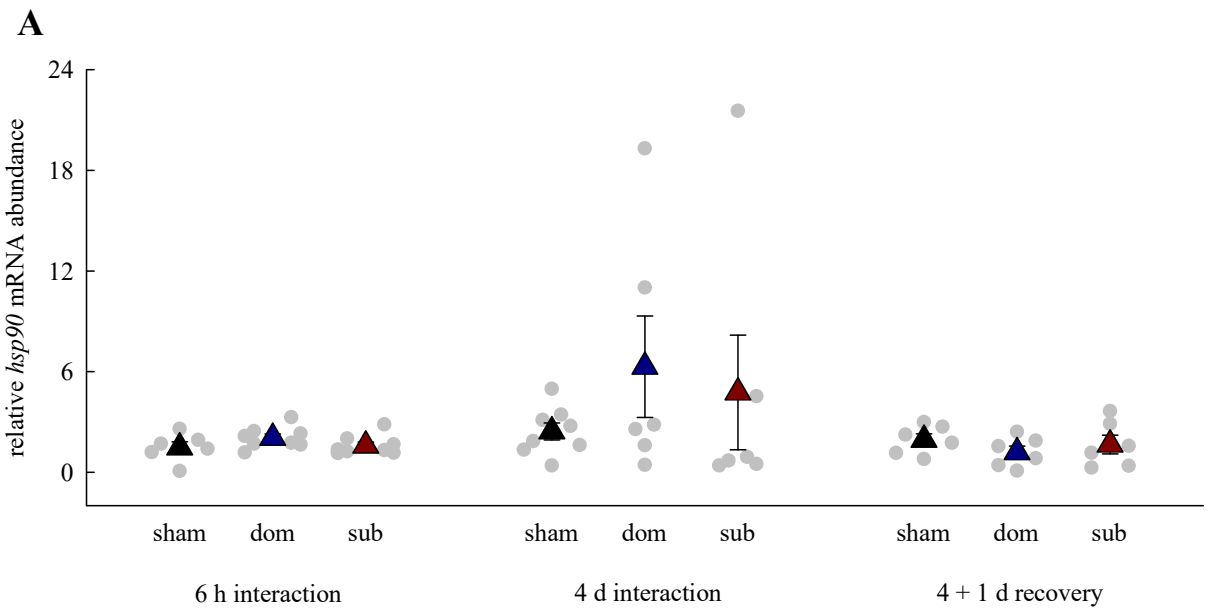

A
ab
ab
ab
ab
a
ab
ab
b
ab
16
12
8
4
0
relative *fkbp4* mRNA abundance
sham
dom
sub
sham
dom
sub
sham
dom
sub
6 h interaction
4 d interaction
4 + 1 d recovery
B
bc
a
ab
abc
abc
abc
abc
c
abc
60
50
40
30
20
10
0
relative *fkbp4* mRNA abundance
sham
dom
sub
sham
dom
sub
sham
dom
sub
6 h interaction
4 d interaction
4 + 1 d recovery

Figure 2.5: Transcript abundance of *fkbp4* in the POA **(A)** and head kidney **(B)** in sham, dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of interaction, or 4 d of interaction followed by 1 d of recovery with the divider replaced in the tank between the two fish. Data are presented as means ± SEM (n = 5-8 per group), with the grey circles representing values for individual fish. Groups that share a letter are not significantly different from one another (2-way ANOVA, **A**: $P_{time} = 0.01$, $P_{status} = 0.38$, $P_{time\ x\ status} = 0.04$; **B:** $P_{time} < 0.001$, $P_{status} = 0.20$, $P_{time\ x\ status} = 0.01$).


A
relative hsp90 mRNA abundance
24
18
12
6
0
sham
dom
sub
sham
dom
sub
sham
dom
sub
6 h interaction
4 d interaction
4 + 1 d recovery

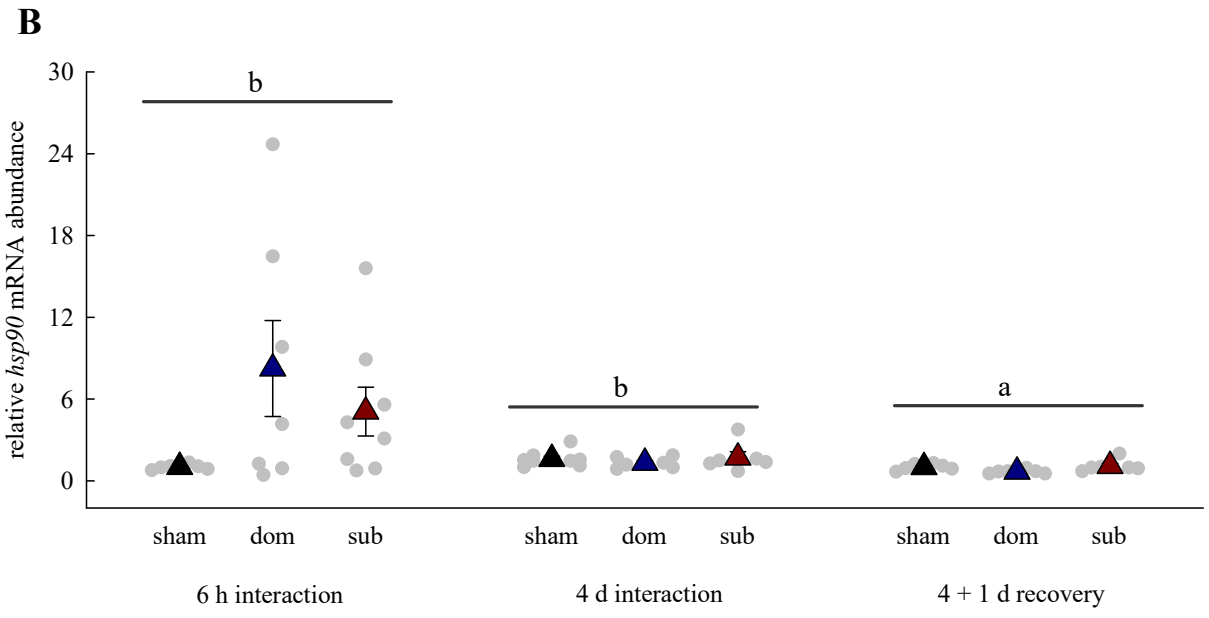

B
relative hsp90 mRNA abundance
30
24
18
12
6
0
b
b
a
sham
dom
sub
sham
dom
sub
sham
dom
sub
6 h interaction
4 d interaction
4 + 1 d recovery

Figure 2.6: Transcript abundance of *hsp90* in the POA **(A)** and head kidney **(B)** in sham, dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of interaction, or 4 d of interaction followed by 1 d of recovery with the divider replaced in the tank between the two fish. Data are presented as means ± SEM (n = 5-8 per group), with the grey circles representing values for individual fish. In panel A, no significant effects were detected whereas in panel B, interaction times that share a letter are not significantly different from one another (**A:** 2-way ANOVA, P_{time} =0.35, P_{status} = 0.42, $P_{time\ x\ status}$ = 0.46; **B:** 2-way ANOVA on ranks, $P_{time} < 0.001$, P_{status} = 0.29, $P_{time\ x\ status}$ = 0.06).

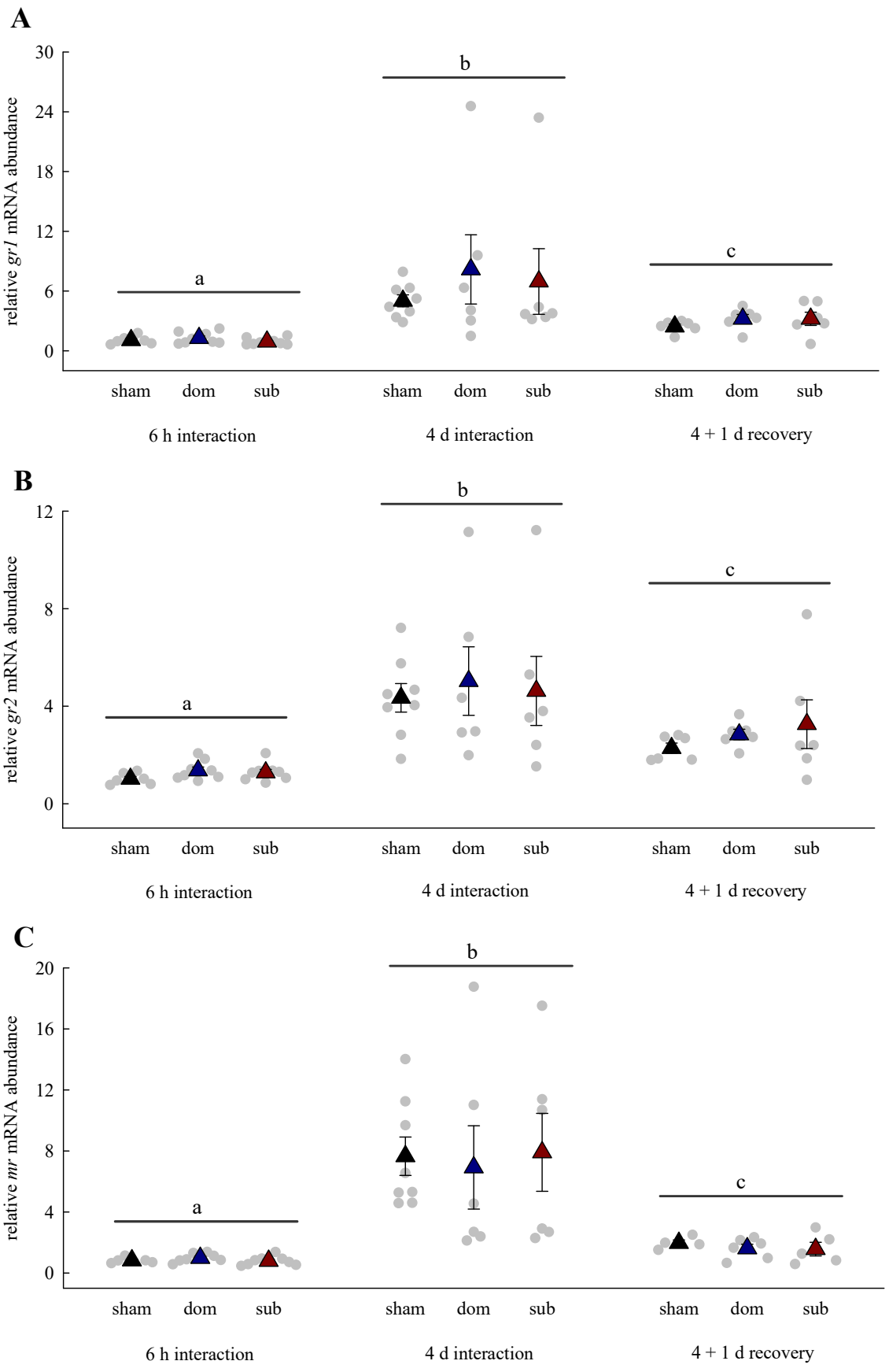

A
relative gr1 mRNA abundance
30
24
18
12
6
0
a
b
c
sham
dom
sub
6 h interaction
4 d interaction
4 + 1 d recovery
B
relative gr2 mRNA abundance
12
8
4
0
a
b
c
sham
dom
sub
6 h interaction
4 d interaction
4 + 1 d recovery
C
relative mr mRNA abundance
20
16
12
8
4
0
a
b
c
sham
dom
sub
6 h interaction
4 d interaction
4 + 1 d recovery

Figure 2.7: Transcript abundance of *gr1* **(A)**, *gr2* **(B)**, and *mr* **(C)** in the POA of sham, dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of interaction, or 4 d of interaction followed by 1 d of recovery with the divider replaced in the tank between the two fish. Data are presented as means ± SEM (n = 6-8 per group), with the grey circles representing values for individual fish. Interaction times that share a letter are not significantly different from one another (2-way ANOVA, **A:** $P_{time} < 0.001$, $P_{status} = 0.44$, $P_{time\ x\ status} = 0.93$; **B:** $P_{time} < 0.001$, $P_{status} = 0.31$, $P_{time\ x\ status} = 0.67$; **C:** $P_{time} < 0.001$, $P_{status} = 0.33$, $P_{time\ x\ status} = 0.61$).

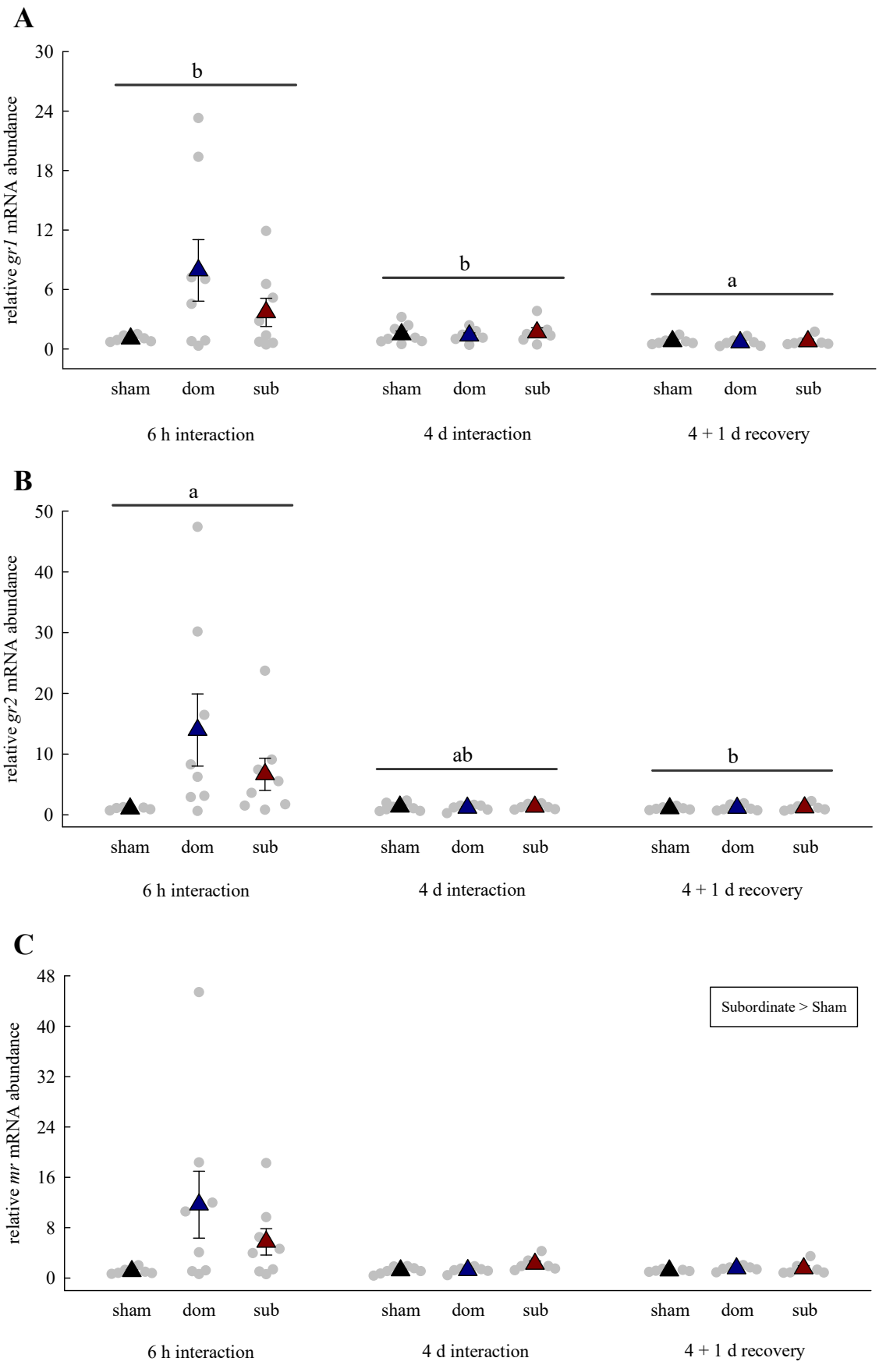

A
relative gr1 mRNA abundance
30
24
18
12
6
0
b
b
a
sham
dom
sub
6 h interaction
4 d interaction
4 + 1 d recovery
B
relative gr2 mRNA abundance
50
40
30
20
10
0
a
ab
b
C
relative mr mRNA abundance
48
40
32
24
16
8
0
Subordinate > Sham

Figure 2.8: Transcript abundance of *gr1* **(A)**, *gr2* **(B)**, and *mr* **(C)** in the head kidney in sham, dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of interaction, or 4 d of interaction followed by 1 d of recovery with the divider replaced in the tank between the two fish. Data are presented as means ± SEM (n = 6-8 per group), with the grey circles representing values for individual fish. Interaction times that share a letter are not significantly different from one another, and significant effects of social status are noted on the figure (**A:** 2-way ANOVA, P_{time} = 0.001, P_{status} = 0.80, $P_{time\ x\ status}$ = 0.51; **B:** 2-way ANOVA, 2-way ANOVA on ranks, P_{time} = 0.01, P_{status} = 0.37, $P_{time\ x\ status}$ = 0.63; **C:** 2-way ANOVA, P_{time} = 0.05, P_{status} = 0.02, $P_{time\ x\ status}$ = 0.19).

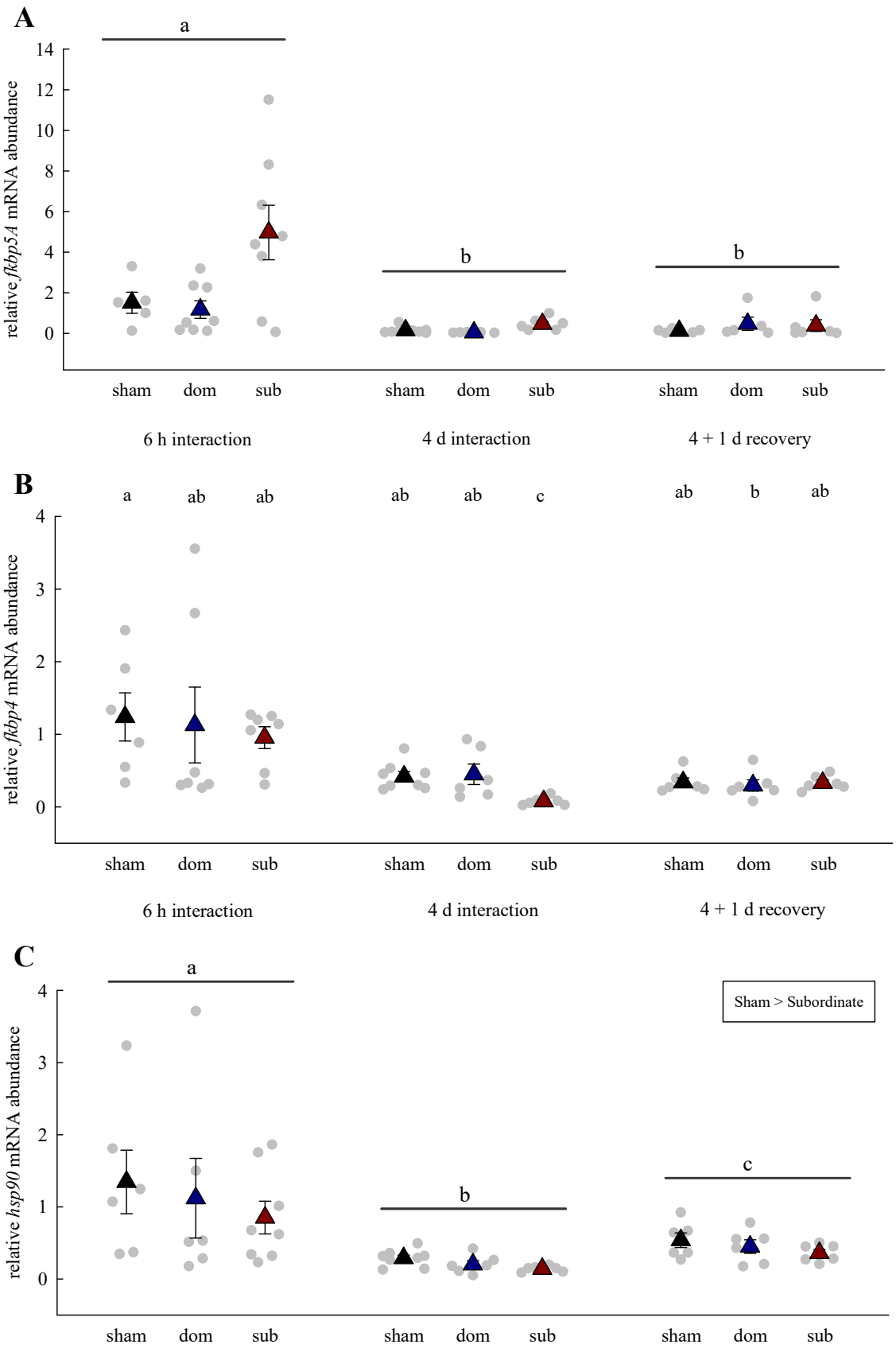

A
a
b
b
14
12
10
8
6
4
2
0
relative *fkbp5A* mRNA abundance
sham
dom
sub
6 h interaction
4 d interaction
4 + 1 d recovery
B
a
ab
ab
ab
ab
c
ab
b
ab
4
3
2
1
0
relative *fkbp4* mRNA abundance
C
a
b
c
Sham > Subordinate
relative *hsp90* mRNA abundance

Figure 2.9: Transcript abundance of *fkbp5A* **(A)**, *fkbp4* **(B)**, and *hsp90* **(C)** in the liver of sham, dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of interaction, or 4 d of interaction followed by 1 d of recovery with the divider replaced in the tank between the two fish. Data are presented as means ± SEM (n = 5-8 per group), with the grey circles representing values for individual fish. In panels A and C, interaction times that share a letter are not significantly different from one another, and significant effects of social status are noted on the figure, whereas in panel B, groups that share a letter are not significantly different from one another (2-way ANOVA, **A:** $P_{time} < 0.001$, $P_{status} = 0.05$, $P_{time\ x\ status} = 0.10$; **B:** $P_{time} < 0.001$, $P_{status} = 0.009$, $P_{time\ x\ status} < 0.001$; **C:** $P_{time} < 0.001$, $P_{status} = 0.047$, $P_{time\ x\ status} = 0.89$).

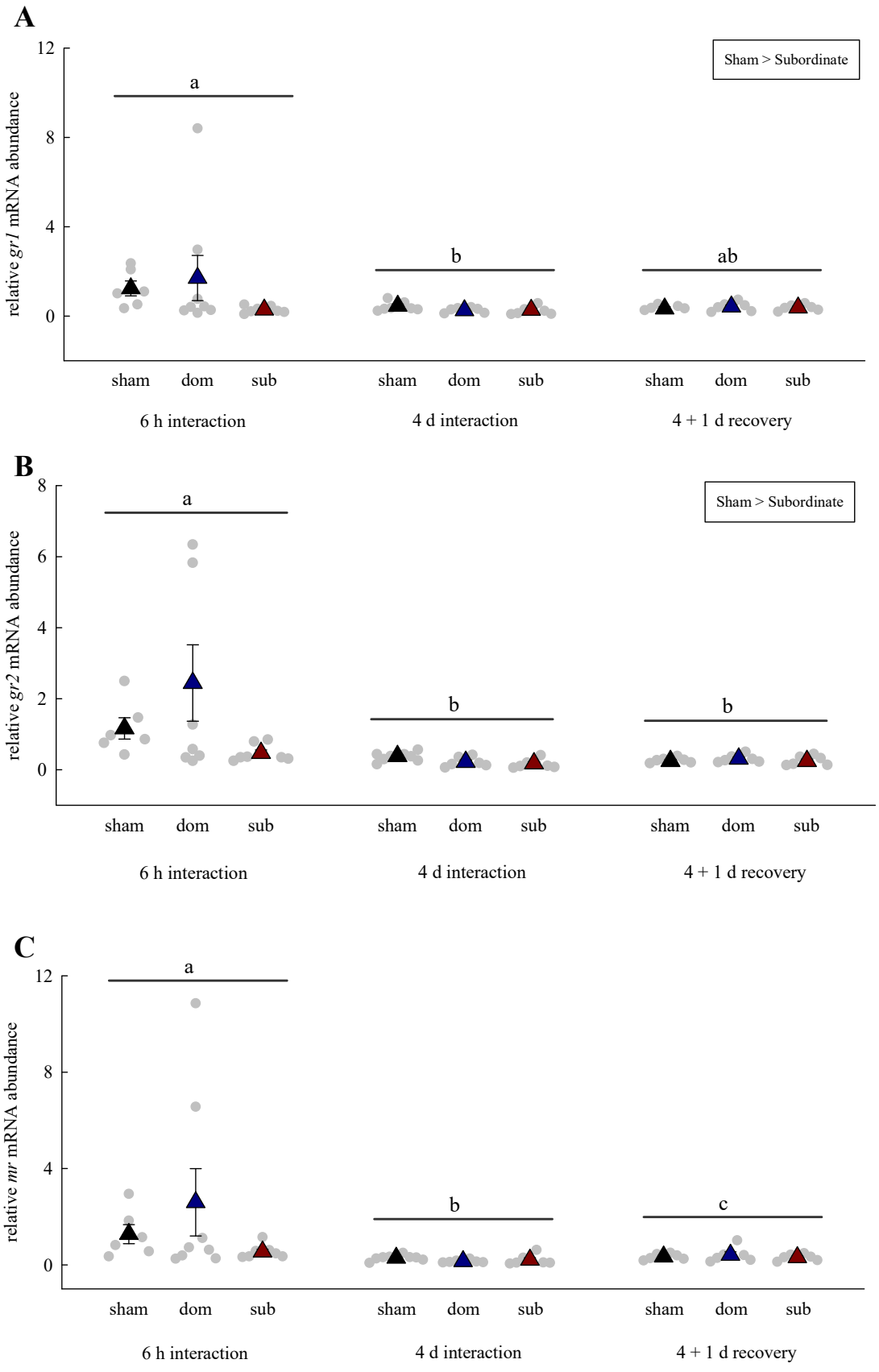

A
relative gr1 mRNA abundance
12
8
4
0
Sham > Subordinate
a
b
ab
sham
dom
sub
6 h interaction
4 d interaction
4 + 1 d recovery
B
relative gr2 mRNA abundance
8
6
4
2
0
Sham > Subordinate
a
b
b
sham
dom
sub
6 h interaction
4 d interaction
4 + 1 d recovery
C
relative mr mRNA abundance
12
8
4
0
a
b
c
sham
dom
sub
6 h interaction
4 d interaction
4 + 1 d recovery

Figure 2.10: Transcript abundance of *gr1* **(A)**, *gr2* **(B)**, and *mr* **(C)** in the liver of sham, dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*) after 6 h or 4 d of interaction, or 4 d of interaction followed by 1 d of recovery with the divider replaced in the tank between the two fish. Data are presented as means ± SEM (n = 5-8 per group), with the grey circles representing values for individual fish. Interaction times that share a letter are not significantly different from one another, and significant effects of social status are noted on the figure (2-way ANOVA, **A:** $P_{time} = 0.03$, $P_{status} = 0.003$, $P_{time\ x\ status} = 0.28$; **B:** $P_{time} < 0.001$, $P_{status} = 0.003$, $P_{time\ x\ status} = 0.25$; **C:** $P_{time} < 0.001$, $P_{status} = 0.06$, $P_{time\ x\ status} = 0.59$).

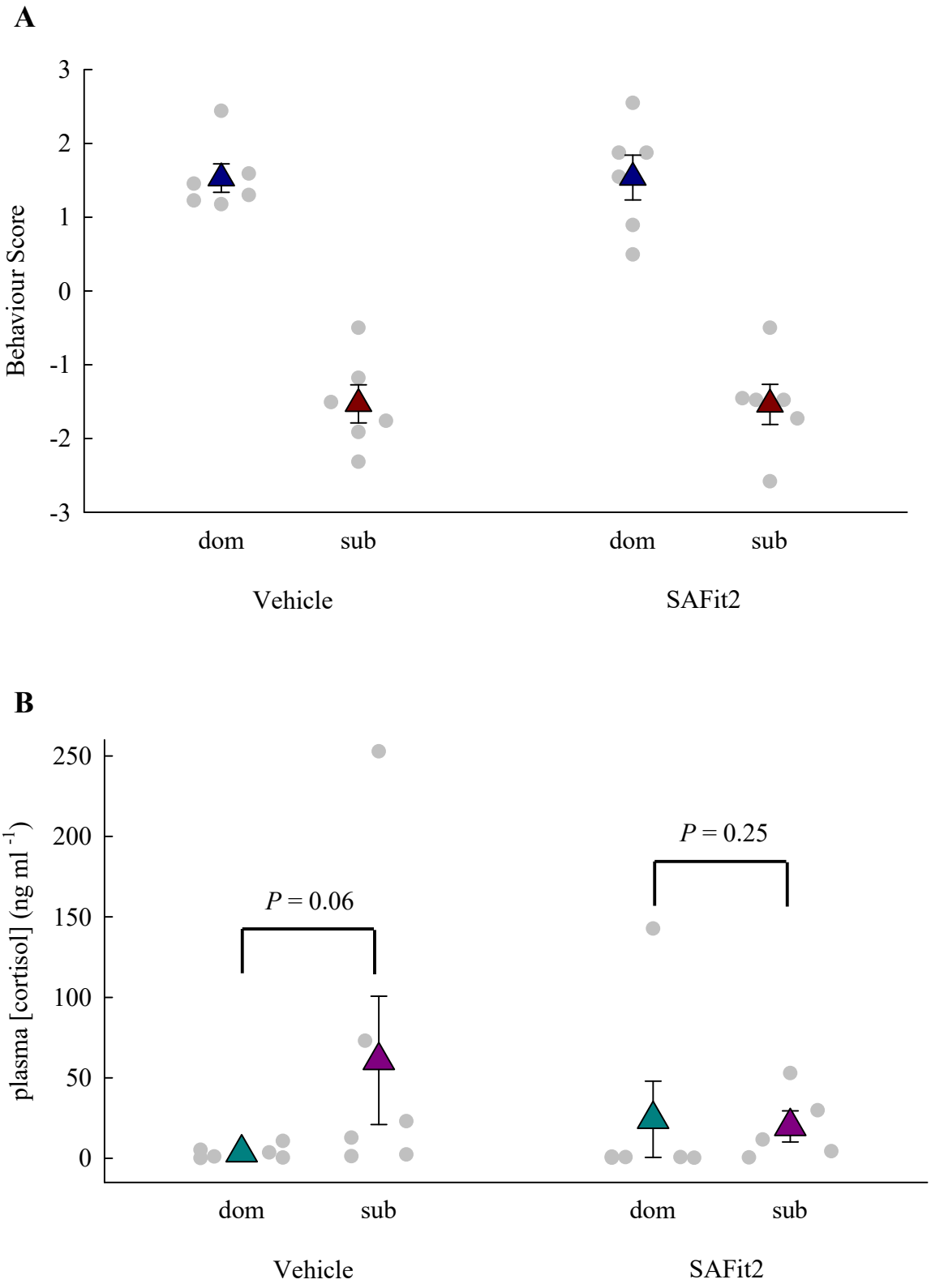

A
Behaviour Score
3
2
1
0
-1
-2
-3
dom
sub
dom
sub
Vehicle
SAFit2
B
plasma [cortisol] (ng ml -1)
250
200
150
100
50
0
P = 0.06
P = 0.25
dom
sub
dom
sub
Vehicle
SAFit2

Figure 2.11: Behaviour scores (**A**) and plasma cortisol concentrations (**B**) for dominant (dom) and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*), where subordinates were injected with vehicle or SAFit2 after 2 d of a 4 d social interaction period. In both panels, data are presented as means ± SEM (n = 5-6 per group), with the grey circles representing values for individual fish. (Wilcoxon rank sum exact test, $P_{dom\ vs\ vehicle\ sub} = 0.06$, $P_{dom\ vs\ SAFit2\ sub} = 0.25$, $P_{vehicle\ sub\ vs\ SAFit2\ sub} = 0.66$).

Table 2.1: Fish length and mass for rainbow trout used in all experiments.

	Dom	**Sub**	**Sham**
Social hierarchies			
Length (cm)	17.9 ± 0.29 (20)	18.1 ± 0.29 (20)	18.1 ± 0.32 (20)
Mass (g)	67.1 ± 3.42 (20)	67.2 ± 3.45 (20)	68.1 ± 4.03 (20)
Specific growth rate (% day^{-1})	-0.61 ± 0.57 (20)	-0.59 ± 0.64 (20)	-0.75 ± 0.40 (20)
SAFit2 experiment			
Length (cm)	24.7 ± 0.34 (6)	25.6 ± 0.34 (6)	
Mass (g)	180.5 ± 7.92 (6)	178.2 ± 7.95 (6)	
Specific growth rate (% day^{-1})	-0.44 ± 0.26 (6)	-0.42 ± 0.35 (6)	

Values in parentheses represent *n* numbers.

Table 2.2: Gene-specific primers used for real-time RT-PCR.

Gene	Primer sequences (5' to 3')	GenBank Accession Number	Amplicon length (bp)	Efficiency (%)	Source
fkbp5A	F: TGATCTATTGGTTGGGCTGTCT R: TCAGTGTCGCACCACTGTTC	XM_021568816.2	110	POA: 60 HK: 69 L: 100	-
fkbp5B	F: ACCTCCCAATGCCATGCTAC R: GGTGCACTGTGGAATACAGG	XM_036983904.1	164	POA: 100 HK: 104	-
fkbp4	F: AGGTTTGCAGAAAGAAGTTCTCC R: CCTGTGCCCTCTTGCTTCAC	XM_021572140.2	148	POA: 93 HK: 94 L: 97	-
hsp90	F: TCCCTTTCTATTTTCCTGCGT R: CTCCTCTTGGCGCATTTCTTC	XM_021611214.2	116	POA: 99 HK: 100 L: 107	-
gr1	F: TTCCAAGTCCACCACATCAA R: GGAGAGCTCCATCTGAGTCG	NM_001124730.1	115	POA: 92 HK: 99 L: 99	(Johansen et al., 2011)
gr2	F: GGGGTGATCAAACAGGAGAA R: CTCACCCCACAGATGGAGAT	NM_001124482.1	140	POA: 106 HK: 107 L: 107	(Johansen et al., 2011)
mr	F: GGCAGCGTTTGAGGAGATGA R: CATGGCGTCCAGTAGCTTGG	AF209873.1	127	POA: 106 HK: 114 L: 118	(Teles et al., 2013)
klf9	F: ATGTCGGAGGTTGATCTCGC R: GCACAGGGATCCGTTAACCA	XM_021605490.2	258	POA: 103	-
ß-actin	F: AGAGCTACGAGCTGCCTGAC R: GTGTTGGCGTACAGGTCCTT	NM_001124235.1	179	POA: 105 HK: 92 L: 105	(Johansen et al., 2011)

HK, head kidney; *L*, liver; *POA*, preoptic area of the brain

Table 2.3: Summary of transcript abundance changes in subordinate rainbow trout compared to sham and/or dominant fish.

	POA			Head kidney			Liver		
	6 h	**4 d**	**4 + 1 d**	**6 h**	**4 d**	**4 + 1 d**	**6 h**	**4 d**	**4 + 1 d**
fkbp5A	-	-	-	↑	↑	-	-	-	-
fkbp5B	-	↑	-	↑	↑	↑			
klf9	↑	↑	↑						
fkbp4	-	-	-	-	-	-	-	↓	-
hsp90	-	-	-	-	-	-	↓	↓	↓
gr1	-	-	-	-	-	-	↓	↓	↓
gr2	-	-	-	-	-	-	↓	↓	↓
mr	-	-	-	↑	↑	↑	-	-	-

-, no change; ↑, increase; ↓, decrease

Cells are filled in grey where transcript abundance was not measured

Appendix 2.1

Figure A1: Distribution of *fkbp5A* **(A)** and *fkbp5B* **(B)** across tissues in rainbow trout (*Oncorhynchus mykiss*). See Fig. A2 for β-actin distribution. *HK*, head kidney; *HG*, hind gut

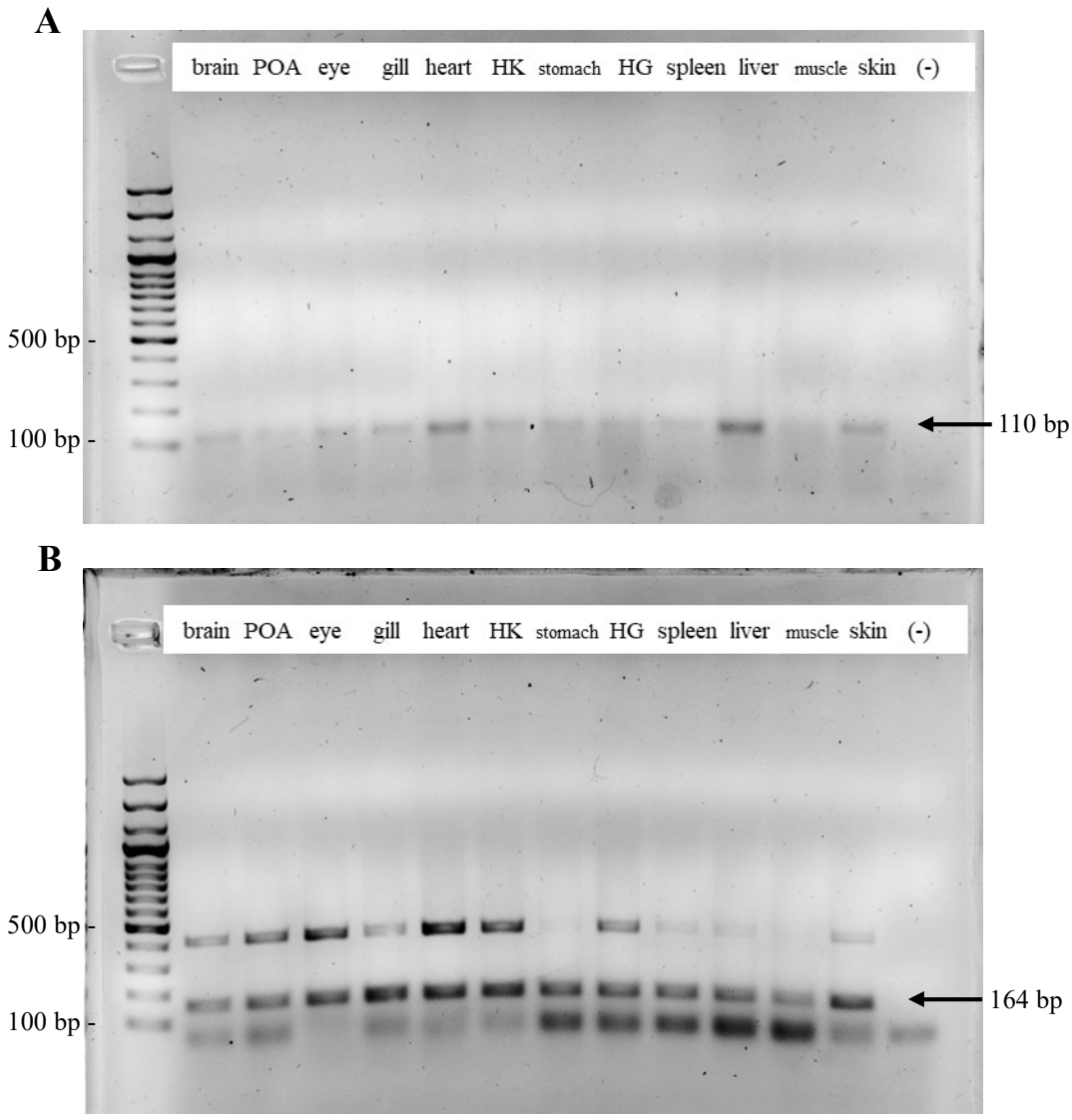
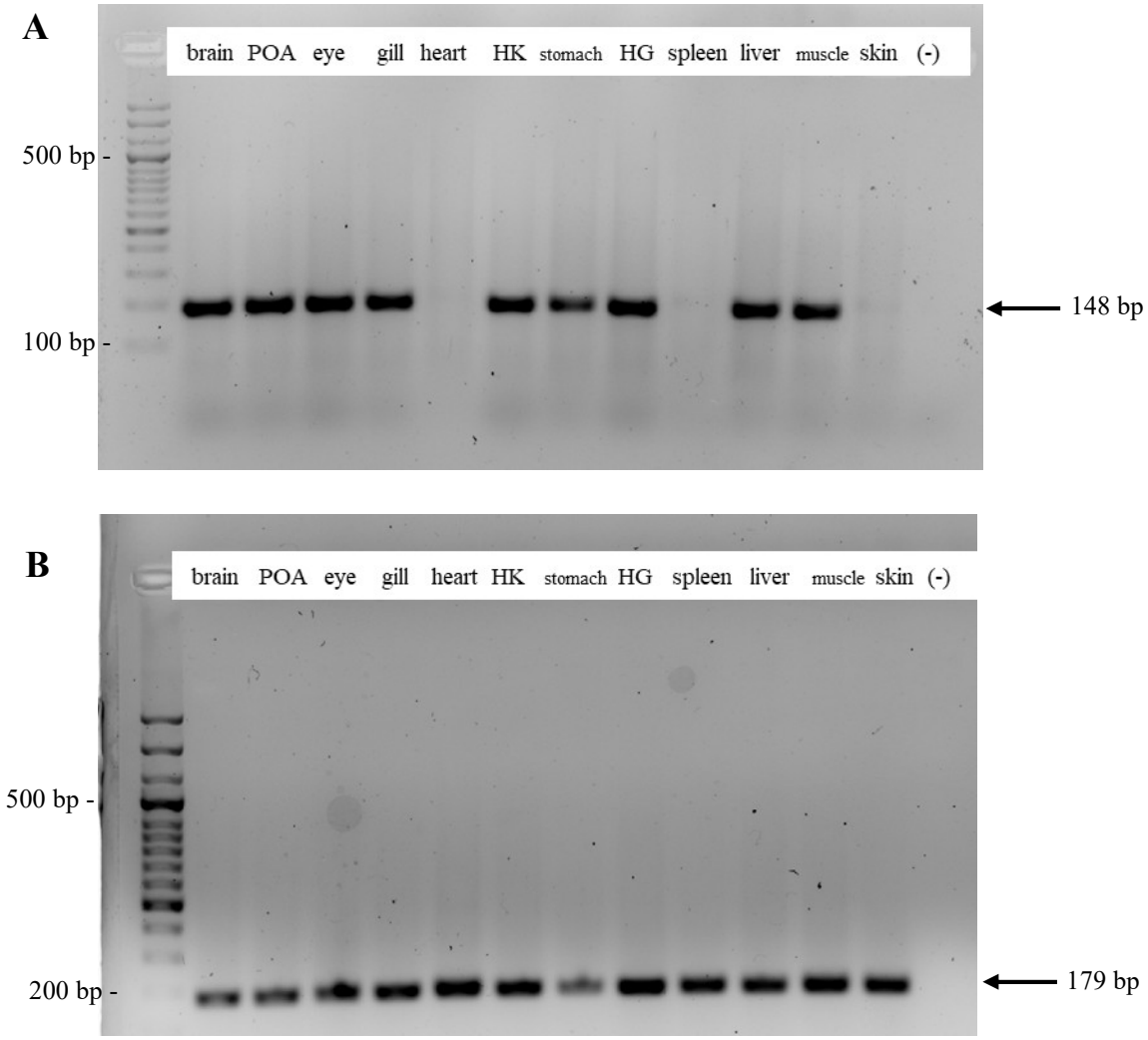


Figure A2: Distribution of *fkbp4* **(A)** and *β-actin* **(B)** across tissues in rainbow trout (*Oncorhynchus mykiss*). *HK*, head kidney; *HG*, hind gut



Chapter 3: Stress responses and *fkbp5* transcript abundance in zebrafish with and without functional glucocorticoid receptors

3.1 Introduction

In teleost fish, the perception of a stressor activates the HPI axis, leading to increased synthesis and circulating concentrations of cortisol, which binds to GR or MR in the cytoplasm of target cells (Faught et al., 2016; Gorissen and Flik, 2016; Zannas et al., 2016). Activation of the GR results in negative feedback inhibition of the HPI axis which serves to return cortisol to baseline levels (Faught et al., 2016; Gorissen and Flik, 2016; Zannas et al., 2016). Studies carried out in GR-KO zebrafish showed that GR is also the main mediator through which cortisol regulates body mass and ion transport (Cruz et al., 2013; Faught et al., 2019).

The stressors experienced by fishes may be acute or chronic, which differ based on the duration of the stressor itself and the breadth of their consequences (Schreck and Tort, 2016). Fish recover quickly from acute stressors, with cortisol concentrations returning to baseline within a few hours or a day, depending on species and water temperature (Schreck and Tort, 2016). However, with a chronic stressor, cortisol may be elevated for a prolonged period (days to weeks) and the capacity of the fish to mount a cortisol response to an additional acute stressor is impaired (Gilmour et al., 2025; Schreck and Tort, 2016). Chronic stress negatively impacts the health of the fish, leading to increased mortality, immunosuppression, alteration of metabolism, decreased growth, and inhibition of brain cell proliferation (Faught and Schaaf, 2024; Filby et al., 2010; Gilmour et al., 2025; Sadoul and Vijayan, 2016; Tea et al., 2019). Examples of acute stressors include encountering a predator, being angled, or sudden changes in the environment, such as changes in temperature, O_2 or CO_2 pressures, or light. In a lab setting, a netting/air-exposure protocol can be used to provide a standardized acute stressor (Ramsay et al., 2009; Schreck and Tort, 2016). Chronic stressors encompass social subordination, or environmental stressors such as overcrowding, prolonged hypoxia, and prolonged exposure to high or low

temperatures (Schreck and Tort, 2016; Tea et al., 2019). In a lab setting, "unpredictable chronic stress" protocols are frequently used to achieve the elevation of cortisol characteristic of exposure to a chronic stressor. These involve exposing fish to one of many different stressors, such as being chased with a net, restrained in a small tube, exposed to a predator, or socially isolated, several times a day at unpredictable times, for a total of 1-2 weeks (Piato et al., 2011; Rambo et al., 2017; Yizhu et al., 2026).

The factors that mediate the maintained elevation of cortisol during exposure to chronic stressors, despite the negative feedback that exists to lower circulating cortisol levels, are unclear. One potential contributor to chronic cortisol elevation is FKBP5, which is thought to be involved in the chronic regulation of the stress axis in mammals (Rein, 2016). Because it can inhibit negative feedback regulation of the HPI axis, FKBP5 has been implicated in the prolonged elevation of cortisol during chronic stress. In mammalian models, cortisol regulates the transcription of *fkbp5* via the GR, with increased *fkbp5* transcript abundance in response to the binding of cortisol to the GR (Rein, 2016; Zannas et al., 2016). The resulting increase in FKBP5 protein abundance then favours FKBP5 binding to GR, inhibiting cortisol binding, GR activation, and the translocation of GR to the nucleus (Rein, 2016; Zannas et al., 2016). Therefore, FKBP5 creates an ultra-short negative feedback loop for GR activity, which impairs its role in negative feedback inhibition of the HPI axis (Rein, 2016; Zannas et al., 2016).

To what extent a comparable mechanism acts in fishes remains unclear, with work to date having focused largely on changes in *fkbp5* mRNA abundance in relation to cortisol levels. For example, expression of *fkbp5* was shown to be induced by GR activation in zebrafish and Atlantic salmon (Chatzopoulou et al., 2017; Gans et al., 2021; Hartig et al., 2020; Tsoulia et al., 2025). Treatment of larval zebrafish with cortisol increased both cortisol production and *fkbp5*

mRNA abundance in adulthood (Hartig et al., 2020), and larval zebrafish chronically exposed to glucocorticoids also demonstrated elevated *fkbp5* expression at 6 days post-fertilization (dpf) (Nagpal et al., 2024). These findings on *fkbp5* expression in larval zebrafish highlight a knowledge gap concerning the patterns of expression of *fkbp5* in zebrafish across early development. Maternally-deposited cortisol and *gr* transcripts are present in zebrafish embryos but decline during the earliest stages of development, increasing after hatch through synthesis and transcription, respectively (Alsop and Vijayan, 2008). However, the cortisol response to a stressor only emerges around 3-4 dpf (Alderman and Bernier, 2009; Alsop and Vijayan, 2008). Whether the induction of *fkbp5* by GR is present in larvae from the early stages of development, when zebrafish are using maternal cortisol deposits, or whether it emerges with the development and activation of cortisol responses and the HPI axis remains to be determined.

Although most teleosts possess two paralogues of *gr* owing to the whole genome duplication that occurred in fishes, zebrafish possess only one *gr* (Alsop and Vijayan, 2008; Bury, 2017). This situation has facilitated the development of zebrafish knockout lines lacking functional GR activity (e.g. Facchinello et al., 2017; Faught and Vijayan, 2018; Gans et al., 2020; Ziv et al., 2013). Larval and adult zebrafish lacking functional GR show elevated baseline levels of cortisol, an effect that has been attributed to the absence of negative feedback (Faught and Vijayan, 2018; Faught et al., 2019). Whether *fkbp5* expression is affected by the absence of GR has not been examined in mammals, because mice lacking the GR die shortly after birth (Cole et al.). Therefore, GR-KO zebrafish provide a novel opportunity to examine the relationships among GR, cortisol, and FKBP5. Preliminary results suggested that GR-KO adult zebrafish have much lower mRNA abundance of *fkbp5* in the brain than wildtype (WT) fish (unpublished

observations, D. St-Amant). Low *fkbp5* transcript abundance was also reported in larval zebrafish lacking functional GR activity (Facchinello et al., 2017; Gans et al., 2021).

Knockout of *fkbp5* provides an alternative route to investigate its role. For example, mice lacking functional expression of *fkbp5* show lower corticosterone levels following chronic stress, whereas *fkbp5* knockout had no effect on corticosterone levels under baseline conditions (Hartmann et al., 2012). In zebrafish, CRSIPR/Cas9 technology has been used for F0 gene deletion of *fkbp5* (Li et al., 2023) as well as to create a mutant line lacking *fkbp5* (Wang et al., 2025). There is one paralogue of *fkbp5* in zebrafish, located on the sixth chromosome.

The present chapter aimed to examine the role of FKBP5 in adult WT zebrafish experiencing prolonged cortisol elevation owing to exposure to a chronic stressor. Prolonged hypoxia, high or low temperature exposures, as well as subordinate social status (Bernier and Craig, 2005; Tao et al., 2025; Tea et al., 2019; Vergauwen et al., 2010) were examined as potential chronic stressors. We had previously developed a zebrafish GR-KO line (Hong, 2024), and here, the effects of GR-KO on cortisol responses to an acute netting stressor were assessed in both larval and adult fish. Use of GR-KO and matched WT zebrafish also allowed patterns of *fkbp5* expression during development and in adults to be investigated. Finally, the development of an *fkbp5*-KO line of zebrafish was initiated.

3.2 Materials and methods

3.2.1 Experimental animals

Adult zebrafish, initially purchased from the pet trade (Big Al's, Ottawa, ON, Canada) were maintained at the University of Ottawa aquatics facility. Fish were held in 3 L or 10 L fibreglass tanks supplied with flowing 28 ± 1°C dechloraminated city of Ottawa tap water ("system"

water). The photoperiod was 14:10 h light:dark. Fish were fed a commercial fish diet (GEMMA Micro 300, Skretting, Vancouver, BC, Canada) twice a day.

University of Ottawa wildtype (WT) zebrafish were obtained from the in-house breeding program. Adult, male, WT zebrafish aged 6-12 months were used for experiments (Table 3.1). Experiments were also carried out on adult (12-18 months) male zebrafish lacking functional expression of the GR (GR-KO; *nr3c1*$^{-/-}$) together with matched WT fish (mWT; *nr3c1*$^{+/+}$) (Table 3.1). Male fish were used for these experiments because female GR-KO fish were needed for breeding purposes. The GR-KO fish were generated through a full gene deletion of NP_001018547, using CRISPR/Cas9 gene-editing technology (Hong, 2024). Four CHOPCHOP-designed guide RNAs (gRNA) and short-guide oligos (sgRNA) that flanked both ends of the target gene were synthesized using a cloning-free method (Talbot and Amacher, 2014). At the one-cell stage, the gRNA was microinjected into *roy*$^{-/-}$; *nacre*$^{-/-}$ (*casper*) embyros. Founder fish carrying the *nr3c1*$^{-/-}$ gene deletion were identified and crossed with WT fish to obtain heterozygous F_1 fish. These were then crossed to generate F_2 populations of GR-KO and mWT fish. Sanger sequencing (Genome Quebec, McGill University, Montreal, QC, Canada) was used to confirm genotypes.

Larval zebrafish were obtained by breeding two male and three female zebrafish in an acrylic breeding trap containing a perforated bottom insert. Embryos raised to 2 or 7 dpf were transferred to 3 L tanks supplied with flowing, system water and held in the laboratory. Embryos raised to 14 dpf were held in system water in Petri dishes in an incubator at 28.5°C until ~24 hours post-fertilization when they were bleached using a standard protocol and transferred to a new Petri dish. After 5 dpf, all dead embryos and larvae were removed, and the larvae were

transferred to a “nursery” holding system and raised under the same conditions as larvae obtained through in-house breeding, until their use at 14 dpf.

All experimental protocols were approved by an institutional animal care committee (protocol BL-3675, University of Ottawa) and complied with the guidelines of the Canadian Council on Animal Care (CCAC) for the use of animals in research and teaching.

3.2.2 Experiment 2. Developmental time course

Under baseline conditions, larval and adult mWT and GR-KO zebrafish were anesthetized using ice-cold buffered MS-222 (0.72 mg mL^{-1} 3-aminobenzoic acid ethyl ester; 21 mM Tris, pH 7; Sigma-Aldrich). At 2 dpf, pools of 25 larvae (n = 1) were collected. At 7 dpf, pools of 15 larvae (n =1) were collected. At 14 dpf, pools of 10 larvae (n = 1) were collected. In adult fish, whole brains were collected. Brains were flash frozen in lN_2 and all samples were stored at -80°C for later analysis of transcript abundance.

3.2.3 Experiment 2. Exposure of adult and larval zebrafish to an acute stressor

The response of *fkbp5* to an acute stressor was assessed using a well-established netting/air-exposure stressor in zebrafish. The protocol consists of netting the fish and holding it in air for 3 min, allowing it to recover in water for 3 min, then netting the fish a second time and holding it in air for another 3 min (Ramsay et al., 2009). Adult and 7 dpf zebrafish were exposed to this netting stressor, then allowed to recover in system water for 10 min, 30 min, 1 h, or 4 h, at which point they were anesthetized using ice-cold buffered MS-222 (0.72 mg mL) for the collection of blood (adult fish; see below) or to be flash frozen in pools of 25 (larvae; n = 1) for later extraction of cortisol. For larvae, cortisol was extracted using the protocol of (Jeffrey and Gilmour, (2016). In brief, samples were thawed on ice and homogenized in 200 µL of extraction

buffer (Neogen, Lansing, MI, USA). Each sample was extracted three times, using 1 mL of diethyl ether each time (Thermo Fisher Scientific). Following the addition of diethyl ether, the sample was vortexed, incubated at room temperature for 30 min (15 min for the subsequent extractions), centrifuged at 3000 *g* for 5 min, and flash frozen at -80°C for 30 min. The top layer was transferred to a clean 1.5 mL microtube and evaporated using forced air. Finally, the extract was reconstituted in 250 µL of extraction buffer (Neogen) and stored at -80°C for subsequent analysis of cortisol concentrations.

3.2.4 Experiment 3. Exposure of adult zebrafish to chronic stressors

Four treatments were tested in adult zebrafish (n = 6 fish per group) as potential chronic stressors, with each treatment lasting four days. The first was exposure to moderate hypoxia (water PO_2 = 30-40 Torr). Fish were placed individually in transparent cylinders with mesh ends, which in turn were placed in a 10 L tank supplied with flowing system water from an equilibration column. The equilibration column was gassed with a mixture of N_2 in air delivered by a two-channel gas mixer (constructed at the University of Ottawa). Water PO_2 and temperature were monitored using a Firesting O_2 meter (FSO2-C4; Pyroscience, Hubertusstr, Germany) equipped with robust oxygen probes (OXROB10; Pyroscience) and a temperature probe (TSUB21; Pyroscience). The oxygen probe was calibrated using air-saturated water and a solution of 4% anhydrous sodium sulphite (ThermoFisher Scientific). This arrangement prevented fish from engaging in aquatic surface respiration during hypoxia. A corresponding set up, with the equilibration column gassed with air to achieve normoxic water, was used for control fish.

Exposure to either low temperature (18°C) or high temperature (32°C) was also tested. In each case, fish were held in a 3 L tank supplied with flowing system water adjusted to the

appropriate temperature using a series 440 Fotopanel thermostatic mixing valve (POWERS™, Burlington, ON, Canada). Control fish were maintained in 28°C water.

Finally, six pairs of zebrafish were allowed to form dominant-subordinate social hierarchies using well-established protocols (Tea et al., 2019). Zebrafish were lightly anesthetized by immersion in a buffered solution of MS-222 (0.24 mg mL^{-1}) to measure length and mass so that fish with a maximum difference of 5% (for length and mass) were paired. Pairs of fish were placed into an experimental chamber (4.5 L). Initially, the members of a pair were separated by an opaque, perforated divider for an overnight recovery period. The following morning, the divider was removed to allow the fish to interact for four days (Tea et al., 2019). Twice a day, in the morning (10-11 h) and the afternoon (14-15 h), for 5 min each time, the behaviour of each fish was scored based on position in the tank, number of aggressive acts, retreats from aggressive acts, and feeding order. Behaviours indicative of dominance include patrolling the tank, being aggressive towards the tankmate, not retreating from aggression, and being the first fish to take food. Higher scores were attributed to these dominant behaviours and the scores for specific behaviours were averaged over the interaction period and combined using a principal components analysis to obtain a single behaviour score for each fish. The fish with the higher behaviour score was assigned dominant status.

In all trials, following the 4 d exposure period, fish were anesthetized using ice-cold buffered MS-222 (0.72 mg mL^{-1}) and blood was collected for the measurement of cortisol (see below).

3.2.5 Tissue collection for adult zebrafish

Blood was collected from terminally anaesthetized adult zebrafish using the approach of Babaei et al. (2013), which is based on caudal severance with 0.5 mol L^{-1} EDTA as an anti-coagulant. The collected blood was centrifuged at 11 000 *g* for 3 min and the resulting plasma

was stored at -80°C for subsequent analysis of plasma cortisol concentrations. The (whole) brain was also collected from adult zebrafish, flash frozen in lN_2, and stored at -80°C for later analysis of transcript abundance.

3.2.6 Measurement of cortisol

Cortisol concentrations in plasma (adult fish) or in whole-body extracts (larval fish) were measured using an enzyme-linked immunoassay (EIA), according to the manufacturer's instructions (Neogen). Samples were assayed in duplicate and the inter- and intra-assay coefficients of variation averaged 7.8% and 8.2%, respectively.

3.2.7 Measurement of transcript abundance by real-time RT-PCR

Adult, brain tissue was thawed on ice and total RNA was extracted by sonicating with the Fisher Model 120 sonicator (Thermo Fisher Scientific) on ice in TRIzol reagent (Invitrogen). Larvae were homogenized with a handheld Kimble Pellet Pestle tissue grinder (Thermo Fisher Scientific) on ice in TRIzol reagent (Invitrogen). The resultant RNA was suspended in nuclease-free water and a Nanodrop 2000c UV-Vis Spectrophotometer (Thermo Fisher Scientific) was used to quantify the RNA. Genomic DNA was removed with the DNase I, RNase-free kit (Thermo Fisher Scientific), and cDNA synthesis was performed using 1 µg of total RNA with the Applied Biosystems cDNA Reverse Transcription Kit (Thermo Fisher Scientific), according to the manufacturer's instructions.

Real-time RT-PCR was carried out using BlasTaq 2X qPCR Mastermix (Applied Biological Materials) and a CFX Opus 96 real-time PCR machine (Bio-Rad Laboratories, Mississauga, ON, Canada), following the manufacturer's instructions. Primers were identified from the literature or designed using Primer-BLAST (Table 3.2). Real-time RT-PCR reactions were carried out using a

reaction volume of 8 µL, which consisted of 5 µL 2X BlasTaq mix, 0.5 µL forward primer (10 µM), 0.5 µL reverse primer (10 µM), 2 µL nuclease-free water, and 1 µL cDNA template. The PCR cycling conditions consisted of an initial 3-min activation at 95℃, followed by 40 cycles of 10-s denaturation at 95°C, 10-s annealing at 60℃, and 10-s extension at 72℃. Samples were assayed with no-template controls (which used H_2O instead of cDNA as template) and two no-RT controls (cDNA synthesis reaction carried out without reverse transcriptase). To confirm reaction efficiency, a standard curve was run for each primer set separately in larval zebrafish and adult brain (Table 3.2). Transcript abundance of the reference gene, *ß-actin*, was also measured in each tissue, and the stability of its expression across genotype and developmental age was confirmed. The fold-change of the target gene relative to the reference gene was expressed relative to the 2 dpf mWTs for larvae or the baseline mWT for adult brain using the delta-delta Ct method (Pfaffl, 2001).

3.2.8 Development of an fkbp5-KO line

To generate zebrafish lacking functional expression of *fkbp5* (FKBP5-KO, *fkbp5*$^{-/-}$), a full gene deletion of NP_998314.1 was attempted using CRISPR/Cas9 gene-editing technology. Four CHOPCHOP-designed guide RNAs (gRNA) and short-guide oligos (sgRNA) that flanked both ends of the target gene were synthesized using a cloning-free method (Fig. 3.1) (Talbot and Amacher, 2014). These were microinjected into *roy*$^{-/-}$; *nacre*$^{-/-}$ (*casper*) embyros at the one-cell stage. Founder fish carrying the *fkbp5*$^{-/-}$ gene deletion were detected using *casper* embyros and crossed with WT fish to obtain F_1 fish. These fish were genotyped in an effort to find heterozygous F_1 fish that could be crossed to generate an F_2 population (Fig. 3.1).

To extract DNA, 40 µL of 50 mM NaOH was added to tubes containing small clipped portions of the caudal fin. The tubes were heated at 95°C for 30 min, allowed to return to room

temperature, then neutralized with 30 µL of 1 M Tris. The PCR reactions consisted of 5 µL MegaFi Pro Fidelity 2X PCR Mastermix (Applied Biological Materials), 0.5 µL forward primer (10 µM, Table 3.3), 0.5 µL reverse primer (10 µM, Table 3.3), 3 µL nuclease-free water. The PCR cycling conditions consisted of a 5-min activation at 95°C, followed by 40 cycles of a 15-s denaturation at 95°C, 15-s annealing at 60°C, 30-s extension at 72°C, then 5-min at 72°C. The PCR products were visualized on a 1.5% agarose gel stained with DyneNA-View Gel Stain (GeneBio Systems), together with a negative control and a Bio-Helix 50 bp DNA ladder (Froggabio). Potential heterozygous fish were sent out for Sanger sequencing (Genome Quebec).

3.2.9 Statistical analysis

All statistical analyses were conducted using R-Studio (https://posit.co/download/rstudio-desktop/). Data are presented as means ± standard error of the mean (SEM). A Grubb's test was used to identify outliers, which were removed from the data set. For cortisol concentrations and relative mRNA abundance of adults exposed to an acute stress, data were analysed by two-way analysis of variance (ANOVA) using genotype and sampling time as factors. For relative mRNA abundance of each gene in larvae, two-way ANOVA was also used with genotype and stage of development as factors. For adult GR-KO and mWT fish, a Wilcoxon rank sum exact test was used to assess the significance of differences in transcript abundance. Student's *t*-tests or one-way ANOVA were used to test for differences in plasma cortisol in adult fish exposed to potential chronic stressors. Data were transformed where assumptions of normality and equal variance were not met, or an equivalent non-parametric test was used. Differences were considered to be statistically significant when $P < 0.05$.

3.3 Results

Experiment 1 examined the baseline expression of *fkbp5* across development and in relation to the presence or absence of functional GR activity. Because cortisol induces *fkbp5* expression by acting through the GR (Zannas et al., 2016), *fkbp5* transcript abundance was measured in GR-KO fish and mWTs, at 2, 7, and 14 dpf, and in adult fish. At all ages, *fkbp5* mRNA abundance was significantly higher in mWT compared to GR-KO fish, and transcript abundance at all larval stages differed from each other, with no significant interaction between genotype and age (Fig. 3.2). The baseline abundance of *fkbp4* and *hsp90* were also measured at these stages. Transcript abundance of *fkbp4* was not affected by genotype in either larval or adult fish, but mRNA abundance was higher at 2 dpf than at 7 dpf (Fig. 3.3). Transcript abundance of *hsp90* differed significantly between GR-KO and mWT at 2 dpf but not at any other age (Fig. 3.4). In larvae, *hsp90* mRNA abundance was lowest at 7 dpf, with abundance at 14 dpf being intermediate between that at 7 and 2 dpf (Fig. 3.4A).

In Experiment 2, the responses of plasma cortisol and brain *fkbp5* transcript abundance to an acute netting stressor were evaluated in adult GR-KO and mWT fish. In GR-KO fish, baseline plasma cortisol concentrations were elevated compared to levels in mWT fish, and they remained at about the same level across all sampling times post exposure to the netting stressor (Fig. 3.5A). By contrast, baseline cortisol concentrations in mWT fish were low, increased to levels comparable to those in GR-KO fish at 10 min after the netting stressor and declined at later times, approaching but not reaching the baseline level 1 h and 4 h post stressor (Fig. 3.5A). As noted above, baseline *fkbp5* mRNA abundance was elevated in mWT fish compared to GR-KO fish (Fig. 3.2B). Unexpectedly, *fkbp5* transcript abundance was below the level of detection at 10 and 30 min post netting stressor in the brain of both mWT and GR-KO fish, and in GR-KO fish,

this pattern continued at 1 and 4 h post stressor (Fig. 3.5B). In mWT fish, *fkbp5* transcript abundance was higher 1 h post stressor than at baseline or 4 h post stressor (Fig. 3.5B; 1-way ANOVA, $P = 0.002$). Corresponding data sets for 7 dpf larvae remain to be generated.

Experiment 3 examined several potential chronic stressors in WT male zebrafish. Although plasma cortisol levels appeared to be elevated in fish exposed to 4 d of hypoxia, 4 d of low (18°C) or high (32°C) water temperature, or 4 d of subordinate social status, comparable cortisol concentrations were observed in the control group in each case (Fig. 3.6).

To date, screening of the FKBP5 F_1 population has not yielded any heterozygotes (Fig. 3.7).

3.4 Discussion

The present chapter reported preliminary data examining cortisol responses and *fkbp5* transcript abundance in zebrafish. Because few studies to date have studied FKBP5 in fishes, I took advantage of the strengths of zebrafish as a study species – the ease with which they can be bred in a laboratory environment and their rapid development – to generate a time-course of *fkbp5* expression during early development. Reasoning that knockout of GR could provide insight into the regulation of *fkbp5* expression, experiments used both wildtype and GR-KO zebrafish. The transcript abundance of *fkbp5* was significantly lower in GR-KO than mWT zebrafish regardless of the age of the fish examined (2 dpf to adult), a finding that has also been reported for other GR-KO zebrafish lines (Facchinello et al., 2017; Gans et al., 2021). Cortisol is known to exert transcription regulation over *fkbp5* expression by acting via the GR (Chatzopoulou et al., 2017; Gans et al., 2021; Hartig et al., 2020; Tsoulia et al., 2025). The low expression of *fkbp5* in GR-KO zebrafish suggests that transcriptional regulation of *fkbp5* via the GR is important from the earliest stages of development. Female zebrafish deposit cortisol and

gr transcripts into their eggs (Alsop and Vijayan 2008). Transcriptional activation of the embryonic genome begins at about 3 hours post-fertilization (hpf) (reviewed by Wragg and Müller, 2016), *de novo* cortisol synthesis is detectable at hatch, and the cortisol response to a stressor emerges at 3-4 dpf (Alsop and Vijayan, 2008). The lower mRNA abundance of *fkbp5* at 2 dpf versus 7 dpf followed by lower expression at 14 dpf is consistent with higher transcription of this gene during transcriptional activation.

The transcript abundance of *fkbp4* and *hsp90* were also measured during early development and in adult fish. The effects of GR knockout on these genes were less marked than for *fkbp5*, with no significant effect of genotype on transcript abundance of *fkbp4*, and a significant difference in transcript abundance of *hsp90* with genotype only at 2 dpf. Although FKBP4 has received less research attention than FKBP5, it is known to serve as a positive regulator of GR signalling with FKBP4 binding to GR being required for it to translocate into the nucleus to regulate gene expression (Zannas et al., 2016). Treatment of murine AtT-20 corticotropes with the synthetic glucocorticoid dexamethasone decreased *fkbp4* transcript abundance (Kageyama et al., 2021) Although they did not reach statistical significance, there appeared to be trends for *fkbp4* transcript abundance to be lower in GR-KO zebrafish at 2 and 7 dpf, which would be consistent with the findings for murine corticotropes. Similarly, *hsp90* plays a key role in regulation of GR activity, but its expression is primarily regulated by heat-shock factor 1 (Prodromou, 2016).

To analyze the response of *fkbp5* expression to an acute stressor, a standardized netting/air exposure stressor was employed (Ramsay et al., 2009; Schreck and Tort, 2016). As expected from previous work, GR-KO adult zebrafish displayed elevated baseline plasma cortisol concentrations, which appears to result from a lack of negative feedback control (Faught and

Vijayan, 2018; Faught et al., 2019). Unlike mWT zebrafish, which showed significant elevation of plasma cortisol concentrations 10 and 30 min after the stressor with a decline back toward baseline values at 1 and 4 h post stressor, cortisol concentrations in GR-KO fish were unaffected by exposure to the acute stressor. This lack of further elevation in cortisol following a stressor has also been observed in other GR-KO zebrafish lines (Faught & Vijayan 2018; Facchinello et al. 2017), although increased cortisol in response to an acute stressor has also been observed (Faught & Vijayan 2019). Brain transcript abundance of *fkbp5* appeared to decrease at 10 and 30 min post stressor exposure, because thresholds for amplification were not detected in the majority of GR-KO and mWT fish at these times. By 1 h post-stressor, *fkbp5* mRNA abundance was significantly higher than baseline in mWT fish, while remaining essentially undetectable in GR-KO fish. In an acute stress response, cortisol binds to GR and forms the complex that increases the transcription of *fkbp5*, through binding to the three GREs that are present on its promoter (Eachus et al., 2023; Rein, 2016; Zannas et al., 2016). The elevation of *fkbp5* expression at 1 h post stressor is consistent with this expectation. Although the current study appears to be the first to have assessed the time-course of *fkbp5* expression in response to a stressor in zebrafish, increased *fkbp5* transcript abundance in response to a vortex stressor was reported in 5 dpf larvae together with the absence of an *fkbp5* response in GR-KO fish (Facchinello et al., 2017). In rats exposed to a traumatic stressor, expression of *fkbp5* peaked at 2 h post-stressor (McKibben et al., 2025). In addition, injection of the glucocorticoid dexamethasone into the amygdala or PVN of mice led to a significant increase in *fkbp5* mRNA after 4 h, but upregulation began to be detected after 1 h (Scharf et al., 2011). By 4 h post-stressor, brain *fkbp5* transcript abundance in the current study did not differ from the baseline value, which was consistent with the acute nature of the stressor.

To examine the role of FKBP5 in maintaining the elevation of cortisol during chronic stress, a stressor that causes prolonged elevation of cortisol is needed. Prior work suggested that subordinate social status is an effective chronic stressor in zebrafish (Filby et al., 2010; Tea et al., 2019), but that was not the case in the current study. Similarly, although exposure to high (Vergauwen et al., 2010) or low (Tao et al., 2025; Vergauwen et al., 2010) water temperatures elevated cortisol in previous studies, cortisol levels in the present study did not differ between the warm or cool fish and the control group. Exposure to severe hypoxia for 3 d caused elevated cortisol in rainbow trout (Bernier and Craig, 2005) but was not effective in the present study. The apparent elevation of cortisol in control zebrafish in the present study suggests that these fish experienced stress, possibly owing to the transfer from holding conditions to the experimental set-ups. This issue requires further investigation.

The findings of this chapter are limited by the failure (to date) to identify a reliable chronic stressor for zebrafish. However, the current work has contributed to our understanding of *fkbp5* expression during early development in zebrafish as well as to the time-course of the *fkbp5* transcript response to an acute stressor. A limitation of these results is that whole brain rather than POA was used for adult work, increasing the difficulty of detecting responses that are specific to the HPI axis (Bernier et al., 2009). Although the use of GR-KO fish emphasized the key importance of the GR in transcriptional regulation of *fkbp5*, future work should focus on the development of an *fkbp5*$^{-/-}$ line, which would allow for a more focused analysis of the role of FKBP5 in responding to chronic and acute stressors.

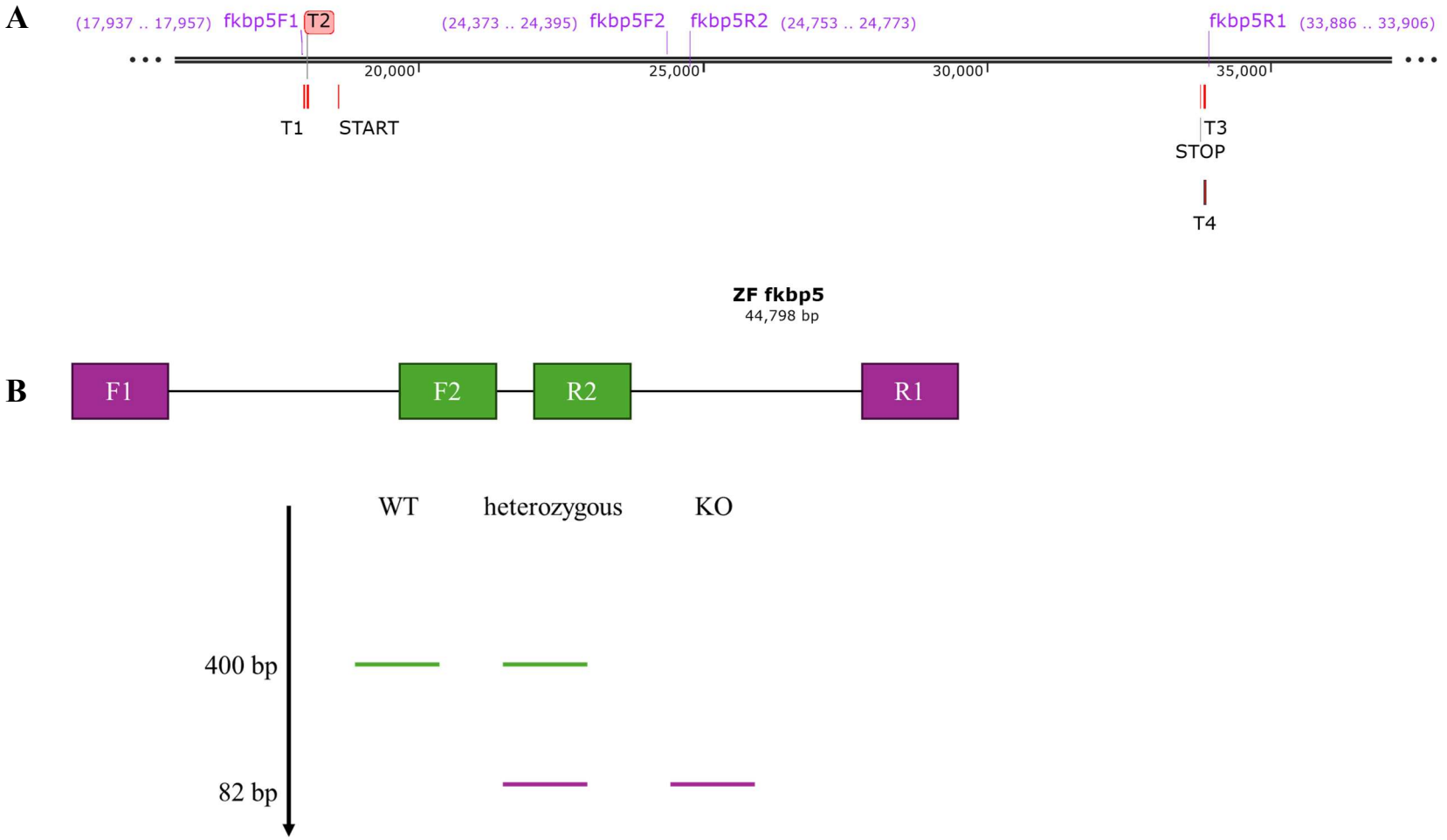


Figure 3.1: SnapGene representation of the *fkbp5* gene deletion through CRISPR/Cas9, showing the target sites (T1-T4) for sgRNA and the binding sites for the screening primers (labelled *fkbp5*F1, F2, R1, R2) **(A),** as well as a schematic of the expected location of bands on an agarose gel for heterozygous and knockout fish based on screening primers **(B)**.

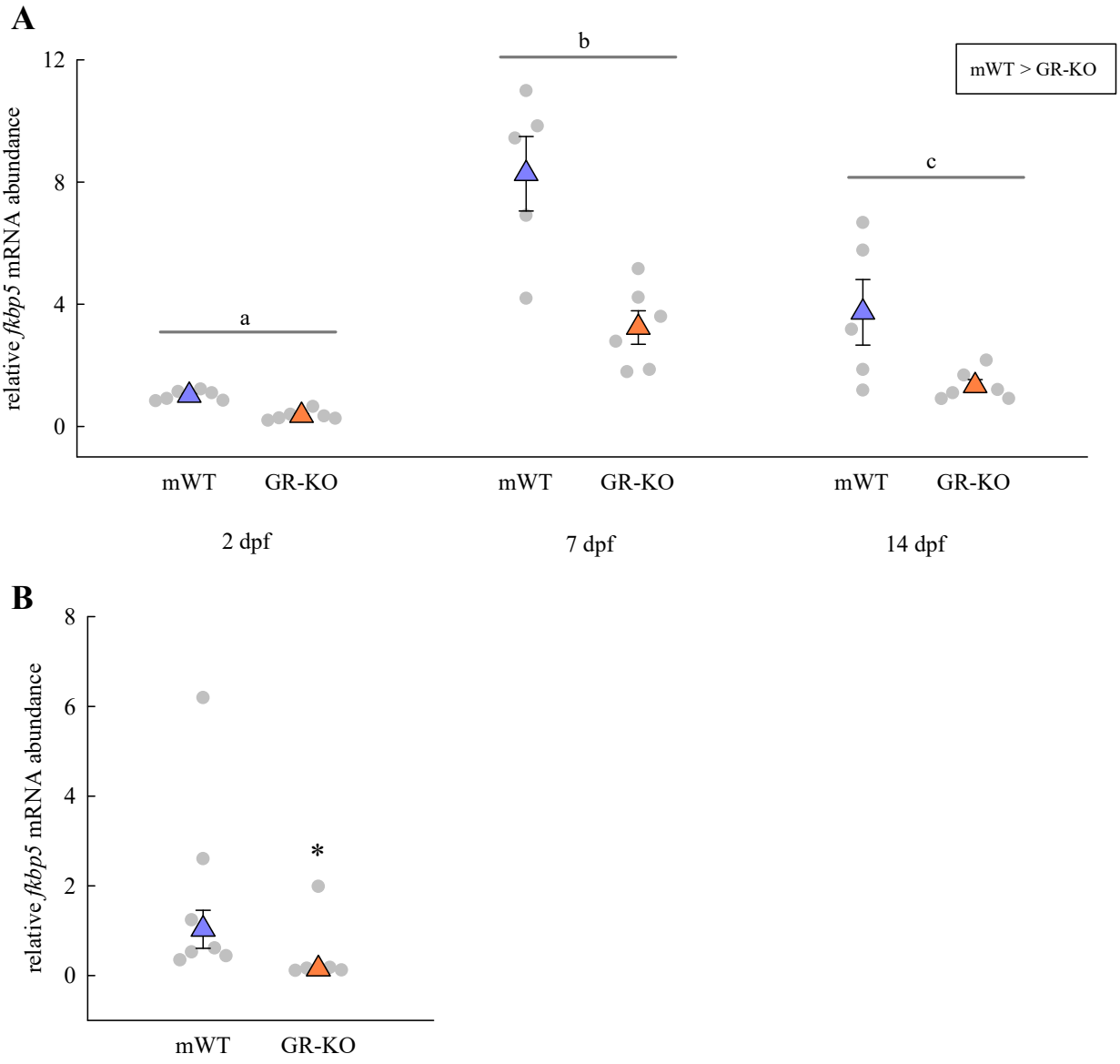


Figure 3.2: Transcript abundance of *fkbp5* in mWT or GR-KO larvae **(A)** and whole brain of adult male, mWT or GR-KO, zebrafish (*Danio rerio*) **(B)** under unstressed (baseline) conditions. Data are presented as means ± SEM (n = 5-7), with the grey circles representing values for individual fish. In panel A, larval ages that share a letter are not different from one another, and significant effects of genotype are indicated on the figure (2-way ANOVA, $P_{genotype} < 0.001$, $P_{age} < 0.001$, $P_{genotype\ x\ age} = 0.34$) and in panel B, significant differences are indicated by an asterisk (Wilcoxon rank sum exact test, $P = 0.006$).

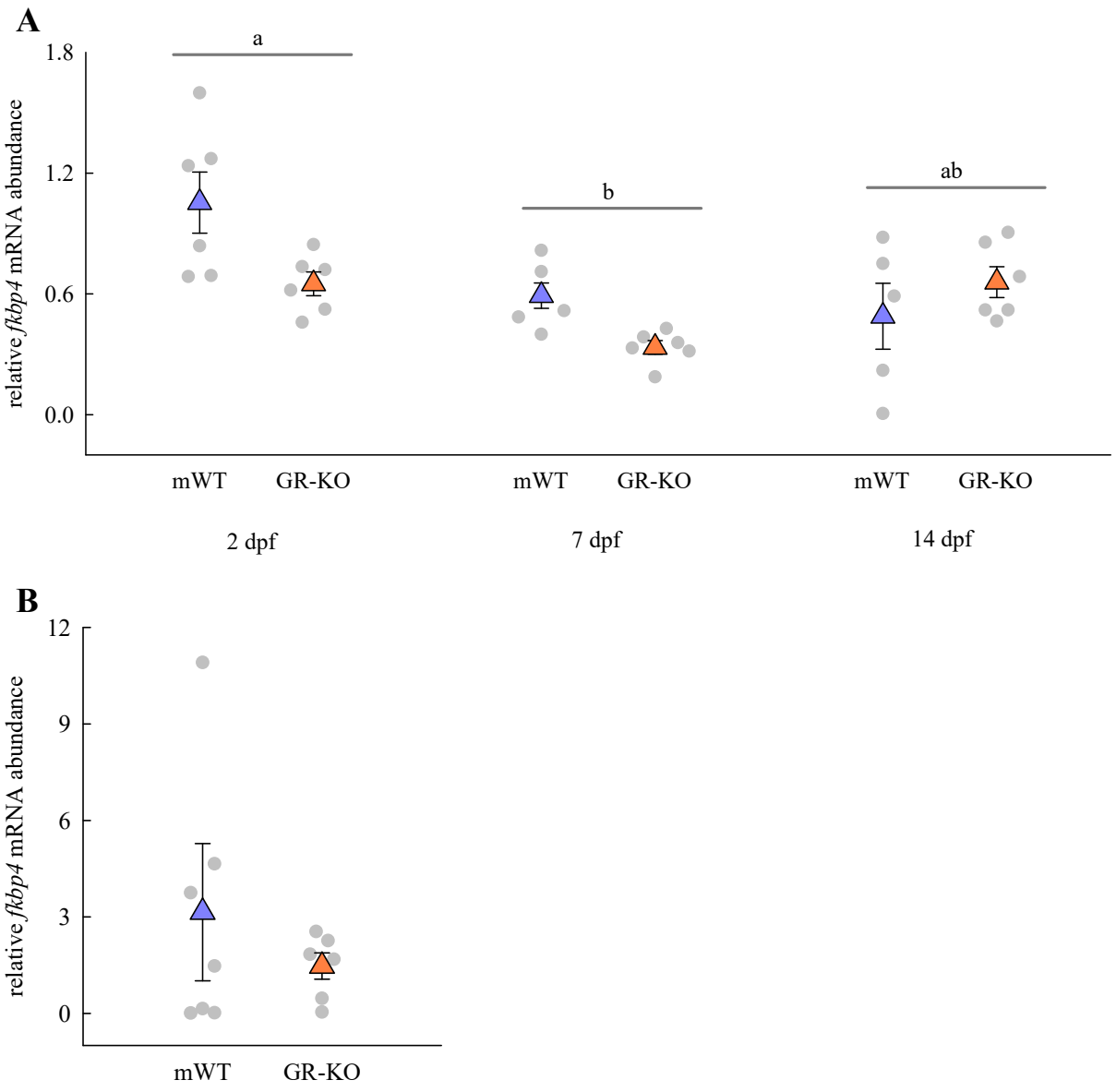


Figure 3.3: Transcript abundance of *fkbp4* in mWT or GR-KO larvae **(A)** and whole brain of adult male, mWT or GR-KO, zebrafish (*Danio rerio*) **(B)** under unstressed (baseline) conditions. In both panels, data are presented as means ± SEM (n = 5-7), with the grey circles representing values for individual fish. In panel A, larval ages that share a letter are not significantly different from one another (2-way ANOVA, $P_{genotype} = 0.09$, $P_{age} = 0.002$, $P_{genotype\ x\ age} = 0.06$). No significant difference between mWT and GR-KO fish was detected in panel B (Wilcoxon rank sum exact test, $P = 1$).

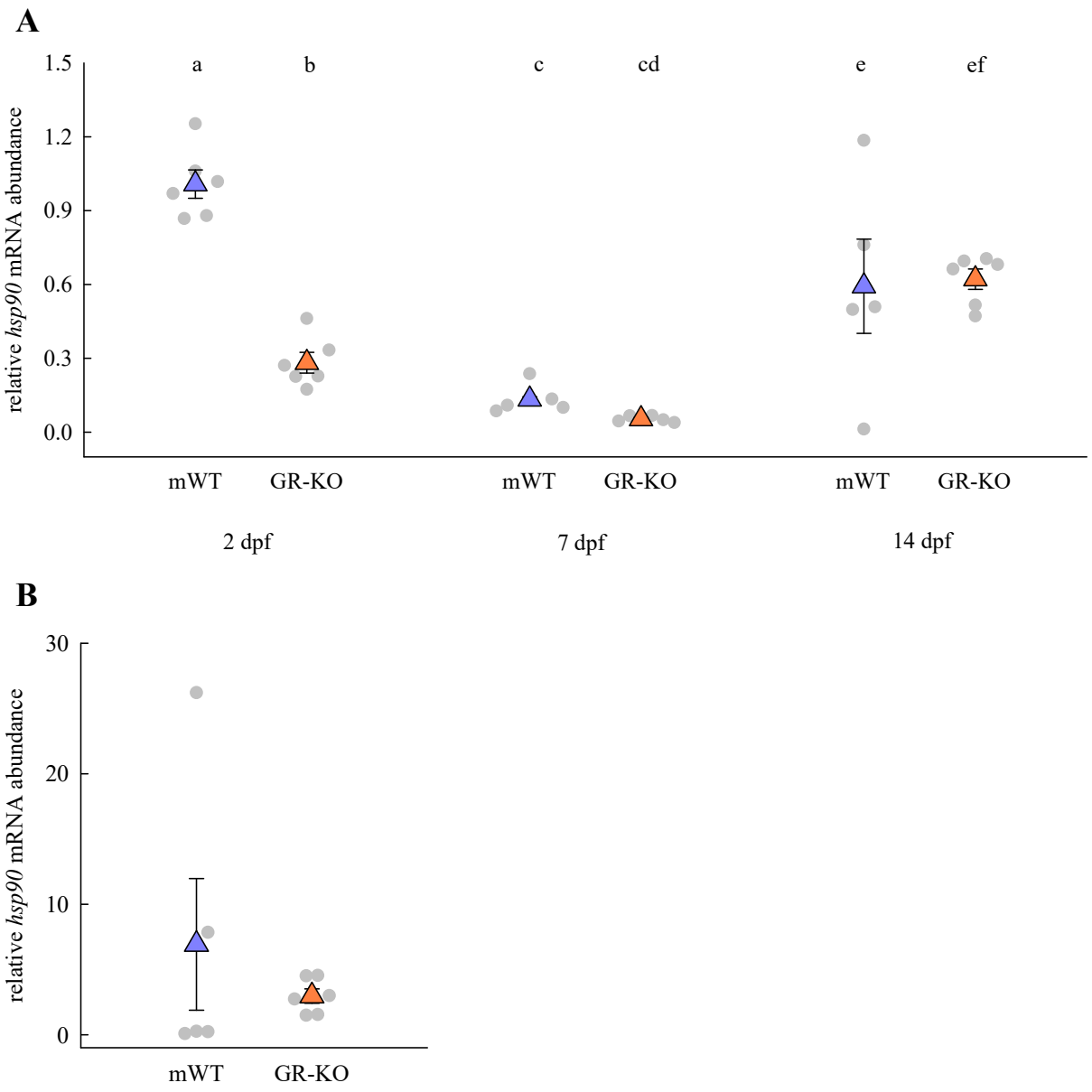


Figure 3.4: Transcript abundance of *hsp90* in mWT or GR-KO larvae **(A)** and whole brain of adult male, mWT or GR-KO, zebrafish (*Danio rerio*) **(B)** under unstressed (baseline) conditions. In both panels, data are presented as means ± SEM (n = 5-7), with the grey circles representing values for individual fish. In panel A, groups that share a letter are not significantly different from one another (2-way ANOVA on ranks, $P_{genotype} = 0.04$, $P_{age} = 0.01$, $P_{genotype\ x\ age} = 0.04$). No significant difference between mWT and GR-KO fish was detected in panel B (Wilcoxon rank sum exact test, $P = 0.66$).

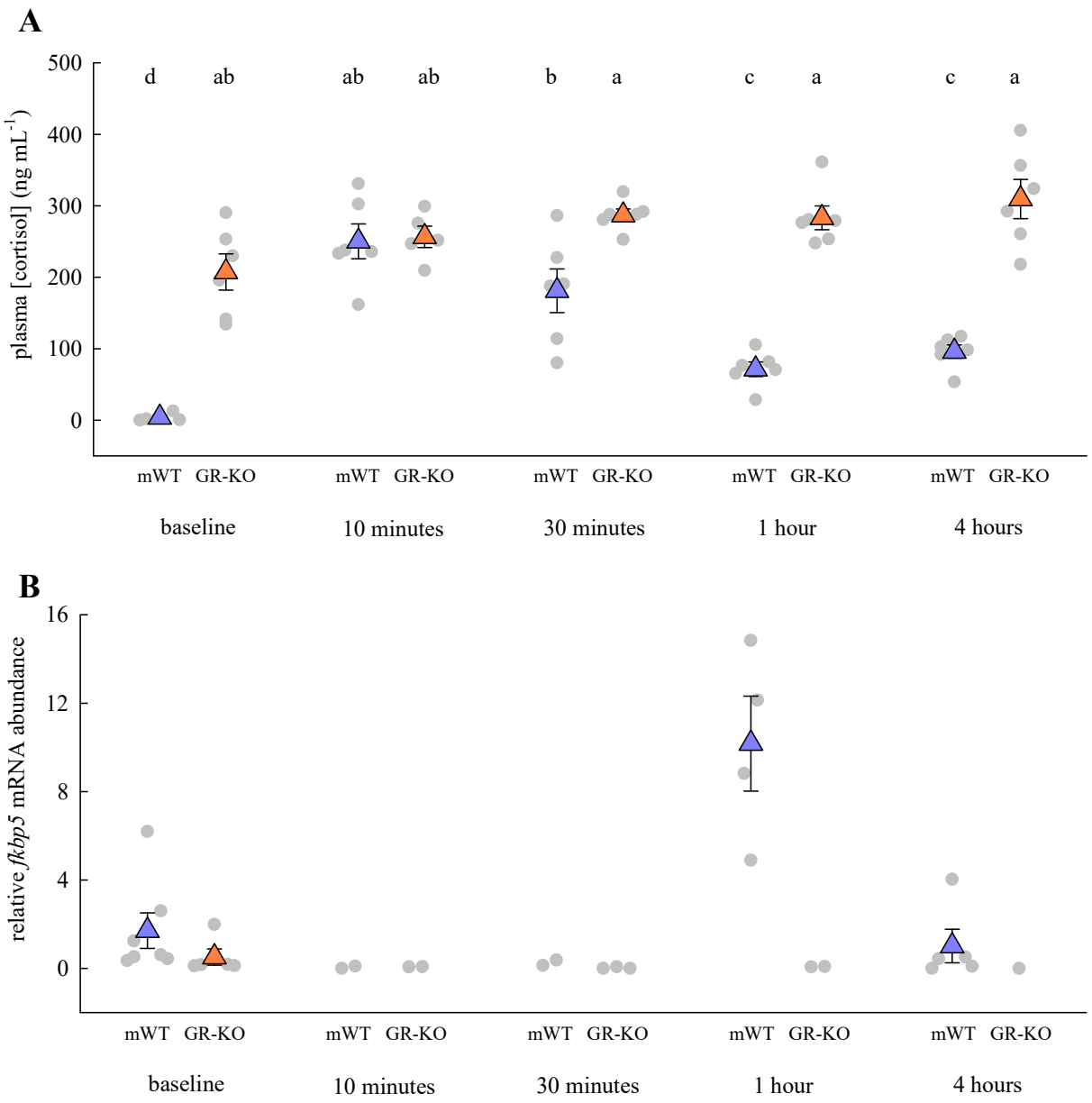


Figure 3.5: Plasma cortisol concentrations **(A)** and whole brain transcript abundance of *fkbp5* **(B)** in mWT or GR-KO male zebrafish (*Danio rerio*) prior to or after being subjected to a netting/air exposure stressor. Data are presented as means ± SEM where n = 4-6, with the grey circles representing values for individual fish. In panel A, groups that share a letter are not significantly different from one another (2-way ANOVA, $P_{genotype} < 0.001$, $P_{time} < 0.001$, $P_{genotype \times time} < 0.001$).

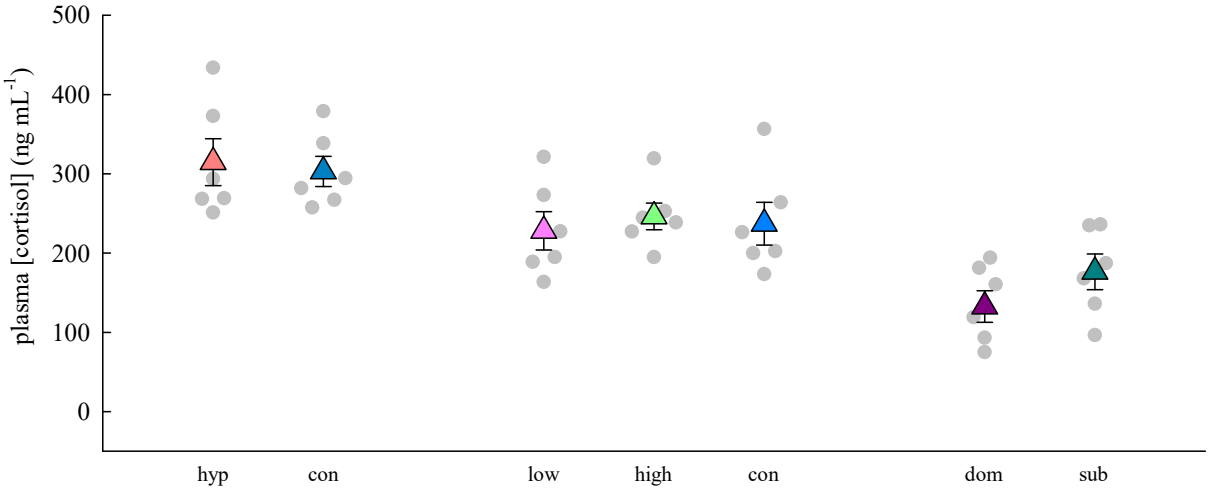


Figure 3.6: Plasma cortisol concentrations in WT male zebrafish (*Danio rerio*) exposed to potential chronic stressors (hypoxia, low or high water temperature, or subordinate social status) for 4 d, together with values for corresponding control (con) fish exposed to normoxic or 28°C water, or of dominant social status. Data are presented as means ± SEM (n = 6), with the grey circles representing values for individual fish. No significant differences were detected between 'stressed' fish and their corresponding controls (hypoxia: Student's t-test, P = 0.75; temperature: ANOVA, P = 0.86; social stress: Student's t-test, P = 0.22).

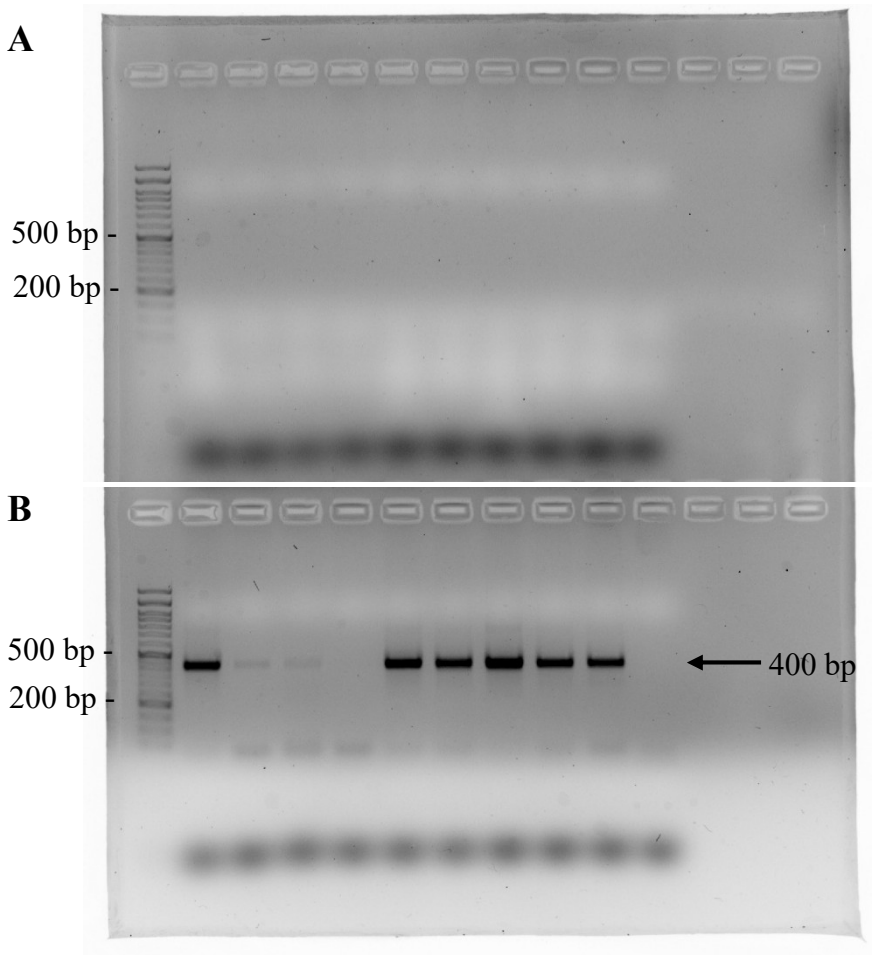


Figure 3.7: 1.5% agarose gel of caudal fin-clipped *fkbp5*$^{-/-}$ F_1 zebrafish (*Danio rerio*) after PCR with F1/R1 primers **(A)**, and 1.5% agarose gel of caudal fin-clipped *fkbp5*$^{-/-}$ F_1 zebrafish after PCR with F2/R2 primers **(B).**

Table 3.1: Fish length and mass for zebrafish used in all experiments.

Experiment	Treatment Groups	Length (cm)	Mass (g)
Acute Stressor	mWT (*n = 30*)	3.3 ± 0.1	0.31 ± 0.02
	GR-KO (*n = 30*)	3.9 ± 0.1	0.46 ± 0.03
Chronic Stressor	Hypoxia (*n* = 12)	3.2 ± 0.04	0.38 ± 0.01
	Temperature (*n* = 18)	3.2 ± 0.1	0.37 ± 0.02
	Dominant-subordinate social 84iérarchies (*n = 12*)	2.8 ± 0.2	0.51 ± 0.22

Table 3.2: Gene specific primers used for real-time RT-PCR.

Gene	Primer sequences (5' to 3')	GenBank Accession Number	Amplicon length (bp)	Efficiency (%)	Source
fkbp5	F: TCTGCCAGCACAAGATTCGTGAGC R: GACCCTGCTTATTCTGATCGGAAA	NM_213149.1	150	Larvae: 102 Whole Brain: 85	(He et al., 2020)
fkbp4	F: TATGCATACGGAGCTGCTGG R: GAGCCCTCCAACCAGACTTC	XM_005173616.6	200	Larvae: 90 Whole Brain: 121	-
gr	F: ACAGCTTCTTCCAGCCTCAG R: CCGGTGTTCTCCTGTTTGAT	NM_001020711.3	116	Larvae: 103 Whole Brain: 105	(Alsop and Vijayan, 2008)
hsp90	F: CTTTCTGCACTCGAGACGAC R: TCAGAGACATGAGCTGAGCG	NM_131328.2	119	Larvae: 100 Whole Brain: 89	-
ß-actin	F: TGTCCCTGTATGCCTCTGGT R: AAGTCCAGACGGAGGATGG	NM_181601.5	121	105	(Alsop and Vijayan, 2008)

Table 3.3: Primers used for PCR assessment of fish genotype.

Primer	**Sequence (5' to 3')**
*fkbp5*F1	CTTGCTTCCTGTAAACCACTG
*fkbp5*F2	CATTGCCTAAAACCTACAGTCAC
*fkbp5*R1	GTTTGTCCGAGAGATGATGAG
*fkbp5*R2	TATTGCATGTGGAGTCACGTG

References

Alderman, S. L. and Bernier, N. J. (2009). Ontogeny of the corticotropin-releasing factor system in zebrafish. *Gen. Comp. Endocrinol.* **164**, 61–69.

Alsop, D. and Vijayan, M. M. (2008). Development of the corticosteroid stress axis and receptor expression in zebrafish. *Am J Physiol Regul Integr Comp Physiol* **294**, 711–719.

Aman, L., Sterlemann, V., Kovarova, V., Narayan, S., Springer, M., Harbich, D., Schmid, B., Jurek, B. and Schmidt, M. V. (2025). The Glucocorticoid Receptor Co-Chaperone FKBP51 in the Adrenal Cortex Is Not Involved in Regulating Hypothalamic–Pituitary–Adrenal Activity in the Mouse. *European Journal of Neuroscience* **62**,.

Babaei, F., Ramalingam, R., Tavendale, A., Liang, Y., Yan, L. S. K., Ajuh, P., Cheng, S. H. and Lam, Y. W. (2013). Novel blood collection method allows plasma proteome analysis from single zebrafish. *J. Proteome Res.* **12**, 1580–1590.

Bernier, N. J. and Craig, P. M. (2005). CRF-related peptides contribute to stress response and regulation of appetite in hypoxic rainbow trout. *Am J Physiol Regul Integr Comp Physiol* **289**, 982–990.

Bernier, N. J., Flik, G. and Klaren, P. H. M. (2009). Chapter 6 Regulation And Contribution Of Corticotropic, Melanotropic And Thyrotropic Axes To The Stress Response In Fishes. *Fish Physiology* **28**, 235–311.

Berthelot, C., Brunet, F., Chalopin, D., Juanchich, A., Bernard, M., Noël, B., Bento, P., Da Silva, C., Labadie, K., Alberti, A., et al. (2014). The rainbow trout genome provides novel insights into evolution after whole-genome duplication in vertebrates. *Nat. Commun.* **5**,.

Best, C. and Gilmour, K. M. (2022). Regulation of cortisol production during chronic social stress in rainbow trout. *Gen. Comp. Endocrinol.* **325**, 114056.

Best, C., Faught, E., Vijayan, M. M. and Gilmour, K. M. (2023). Negative feedback regulation in the hypothalamic-pituitary-interrenal axis of rainbow trout subjected to chronic social stress. *Gen. Comp. Endocrinol.* **341**, 114332.

Best, C., Mennigen, J. A. and Gilmour, K. M. (2024). Exploring transcriptional and post-transcriptional epigenetic regulation of crf and 11βhsd2 in rainbow trout brain during chronic social stress. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **288**,.

Bradford, C. S., Fitzpatrick, M. S. and Schreck, C. B. (1992). Evidence for Ultra-Short-Loop Feedback in ACTH-Induced Interrenal Steroidogenesis in Coho Salmon: Acute Self-Suppression of Cortisol Secretion in Vitro'~~. *Gen. Comp. Endocrinol.* **87**, 292–299.

Brix, L. M., Häusl, A. S., Toksöz, I., Bordes, J., van Doeselaar, L., Engelhardt, C., Narayan, S., Springer, M., Sterlemann, V., Deussing, J. M., et al. (2022). The co-chaperone FKBP51 modulates HPA axis activity and age-related maladaptation of the stress system in pituitary proopiomelanocortin cells. *Psychoneuroendocrinology* **138**,.

Bury, N. R. (2017). The evolution, structure and function of the ray finned fish (Actinopterygii) glucocorticoid receptors. *Gen. Comp. Endocrinol.* **251**, 4–11.

Bury, N. R., Sturm, A., Le Rouzic, P., Lethimonier, C., Ducouret, B., Guiguen, Y., Robinson-Rechavi, M., Laudet, V., Rafestin-Oblin, M. E. and Prunet, P. (2003). Evidence for two distinct functional glucocorticoid receptors in teleost fish. *J. Mol. Endocrinol.* **31**, 141–156.

Chatzopoulou, A., Schoonheim, P. J., Torraca, V., Meijer, A. H., Spaink, H. P. and Schaaf, M. J. M. (2017). Functional analysis reveals no transcriptional role for the glucocorticoid receptor β-isoform in zebrafish. *Mol. Cell. Endocrinol.* **447**, 61–70.

Codagnone, M. G., Kara, N., Ratsika, A., Levone, B. R., van de Wouw, M., Tan, L. A., Cunningham, J. I., Sanchez, C., Cryan, J. F. and O'Leary, O. F. (2022). Inhibition of FKBP51 induces stress resilience and alters hippocampal neurogenesis. *Mol. Psychiatry* **27**, 4928–4938.

Cole, T. J., Blendy, J. A., Monaghan, A. P., Schmid, W., Aguzzi, A. and Schiitz, G. Molecular genetic analysis of glucocorticoid signaling during mouse development. *Steroids* **60**, 93–96.

Cruz, S. A., Lin, C. H., Chao, P. L. and Hwang, P. P. (2013). Glucocorticoid receptor, but not mineralocorticoid receptor, mediates cortisol regulation of epidermal ionocyte development and ion transport in zebrafish (danio rerio). *PLoS One* **8**,.

Cruz, B., Vozella, V., Carper, B. A., Xu, J. C., Kirson, D., Hirsch, S., Nolen, T., Bradley, L., Fain, K., Crawford, M., et al. (2023). FKBP5 inhibitors modulate alcohol drinking and trauma-related behaviors in a model of comorbid post-traumatic stress and alcohol use disorder. *Neuropsychopharmacology* **48**, 1144–1154.

Culbert, B. M. and Gilmour, K. M. (2016). Rapid recovery of the cortisol response following social subordination in rainbow trout. *Physiol. Behav.* **164**, 306–313.

Currie, S., Leblanc, S., Watters, M. A. and Gilmour, K. M. (2010). Agonistic encounters and cellular angst: Social interactions induce heat shock proteins in juvenile salmonid fish. *Proceedings of the Royal Society B: Biological Sciences* **277**, 905–913.

Doyon, C., Gilmour, K. M., Trudeau, V. L. and Moon, T. W. (2003). Corticotropin-releasing factor and neuropeptide Y mRNA levels are elevated in the preoptic area of socially subordinate rainbow trout. *Gen. Comp. Endocrinol.* **133**, 260–271.

Eachus, H., Oberski, L., Paveley, J., Bacila, I., Ashton, J. P., Esposito, U., Seifuddin, F., Pirooznia, M., Elhaik, E., Placzek, M., et al. (2023). Glucocorticoid receptor regulates protein chaperone, circadian clock and affective disorder genes in the zebrafish brain. *DMM Disease Models and Mechanisms* **16**, 1–17.

Ejike, C. and Schreck, C. B. (1980). Stress and Social Hierarchy Rank in Coho Salmon. *Trans. Am. Fish. Soc.* **109**, 423–426.

Elofsson, U. O. E., Mayer, I., Damsgård, B. and Winberg, S. (2000). Intermale competition in sexually mature Arctic charr: Effects on brain monoamines, endocrine stress responses, sex hormone levels, and behavior. *Gen. Comp. Endocrinol.* **118**, 450–460.

Facchinello, N., Skobo, T., Meneghetti, G., Colletti, E., Dinarello, A., Tiso, N., Costa, R., Gioacchini, G., Carnevali, O., Argenton, F., et al. (2017). Nr3c1 null mutant zebrafish are viable and reveal DNA-binding-independent activities of the glucocorticoid receptor. *Sci. Rep.* **7**, 1–13.

Faught, E. and Schaaf, M. J. M. (2024). Molecular mechanisms of the stress-induced regulation of the inflammatory response in fish. *Gen. Comp. Endocrinol.* **345**, 1–9.

Faught, E. and Vijayan, M. M. (2018). The mineralocorticoid receptor is essential for stress axis regulation in zebrafish larvae. *Sci. Rep.* **8**, 1–11.

Faught, E., Aluru, N. and Vijayan, M. M. (2016). The Molecular Stress Response. In *Fish Physiology*, pp. 113–166. Elsevier Inc.

Faught, E., Mathilakath, X. and Vijayan, M. (2019). Loss of the glucocorticoid receptor in zebrafish improves muscle glucose availability and increases growth. *Am J Physiol Endocrinol Metab* **316**, 1093–1104.

Filby, A. L., Paull, G. C., Bartlett, E. J., Van Look, K. J. W. and Tyler, C. R. (2010). Physiological and health consequences of social status in zebrafish (Danio rerio). *Physiol. Behav.* **101**, 576–587.

Fryer, J. N. and Peter, R. E. (1977). Hypothalamic Control of ACTH Secretion in Goldfish III. Hypothalamic Cortisol Implant Studies. *Gen. Comp. Endocrinol.* **33**, 215–225.

Fryer, J., Lederis, K., RIVIERt, J. and RIVIER Cortisol, J. (1984). Cortisol Inhibits the ACTH-Releasing Activity of Urotensin I, CRF and Sauvagine Observed with Superfused Goldfish Pituitary Cells'. *Peptides (N.Y.).* **5**, 925–930.

Gaali, S., Kirschner, A., Cuboni, S., Hartmann, J., Kozany, C., Balsevich, G., Namendorf, C., Fernandez-Vizarra, P., Sippel, C., Zannas, A. S., et al. (2015). Selective inhibitors of the FK506-binding protein 51 by induced fit. *Nat. Chem. Biol.* **11**, 33–37.

Gallo, V. P. and Civinini, A. (2003). Survey of the Adrenal Homolog in Teleosts. *Int. Rev. Cytol.* **230**, 89–187.

Gamperl, A. K., Vijayan, M. M. and Boutilier, R. G. (1994). Experimental control of stress hormone levels in fishes: techniques and applications. *Rev. Fish Biol. Fish* **4**, 215–255.

Gans, I., Hartig, E. I., Zhu, S., Tilden, A. R., Hutchins, L. N., Maki, N. J., Graber, J. H. and Coffman, J. A. (2020). Klf9 is a key feedforward regulator of the transcriptomic response to glucocorticoid receptor activity. *Sci. Rep.* **10**, 1–16.

Gans, I. M., Grendler, J., Babich, R., Jayasundara, N. and Coffman, J. A. (2021). Glucocorticoid-Responsive Transcription Factor Krüppel-Like Factor 9 Regulates fkbp5 and Metabolism. *Front. Cell Dev. Biol.* **9**,.

Gilmour, K. M., DiBattista, J. D. and Thomas, J. B. (2005). Physiological causes and consequences of social status in salmonid fish. *Integr. Comp. Biol.* **45**, 263–273.

Gilmour, K. M., Best, C. and Currie, S. (2025). Using the reactive scope model to redefine the concept of social stress in fishes. *Journal of Experimental Biology* **228**, 1–13.

Gorissen, M. and Flik, G. (2016). The Endocrinology of the Stress Response in Fish: An Adaptation-Physiological View. In *Fish Physiology*, pp. 75–111. Elsevier Inc.

Guidotti, G., Calabrese, F., Anacker, C., Racagni, G., Pariante, C. M. and Riva, M. A. (2013). Glucocorticoid receptor and fkbp5 expression is altered following exposure to chronic stress: Modulation by antidepressant treatment. *Neuropsychopharmacology* **38**, 616–627.

Hartig, E. I., Zhu, S., King, B. L. and Coffman, J. A. (2020). Chronic cortisol exposure in early development leads to neuroendocrine dysregulation in adulthood. *BMC Res. Notes* **13**,.

Hartmann, J. (2025). From intracellular sensors to systemic resilience: Reframing the biology of stress. *Neurobiol. Stress* **39**, 1–5.

Hartmann, J., Wagner, K. V., Liebl, C., Scharf, S. H., Wang, X. D., Wolf, M., Hausch, F., Rein, T., Schmidt, U., Touma, C., et al. (2012). The involvement of FK506-binding protein 51 (FKBP5) in the behavioral and neuroendocrine effects of chronic social defeat stress. *Neuropharmacology* **62**, 332–339.

Hartmann, J., Wagner, K. V., Gaali, S., Kirschner, A., Kozany, C., Rühter, G., Dedic, N., Häusl, A. S., Hoeijmakers, L., Westerholz, S., et al. (2015). Pharmacological inhibition of the psychiatric risk factor FKBP51 has anxiolytic properties. *Journal of Neuroscience* **35**, 9007–9016.

Hartmann, J., Bajaj, T., Klengel, C., Chatzinakos, C., Ebert, T., Dedic, N., McCullough, K. M., Lardenoije, R., Joëls, M., Meijer, O. C., et al. (2021). Mineralocorticoid receptors dampen glucocorticoid receptor sensitivity to stress via regulation of FKBP5. *Cell Rep.* **35**, 1–14.

Häusl, A. S., Brix, L. M., Hartmann, J., Pöhlmann, M. L., Lopez, J. P., Menegaz, D., Brivio, E., Engelhardt, C., Roeh, S., Bajaj, T., et al. (2021). The co-chaperone Fkbp5 shapes the acute stress response in the paraventricular nucleus of the hypothalamus of male mice. *Mol. Psychiatry* **26**, 3060–3076.

He, M., Halima, M., Xie, Y., Schaaf, M. J. M., Meijer, A. H. and Wang, M. (2020). Ginsenoside Rg1 Acts as a Selective Glucocorticoid Receptor Agonist with Anti-Inflammatory Action without Affecting Tissue Regeneration in Zebrafish Larvae. *Cells* **9**,.

Hong, M. *Ion regulation under acidic conditions in zebrafish (Danio rerio) lacking functional corticosteroid receptors*.

Hontela, A., Leblond, V. S. and Chang, J. P. (2008). Purification and isolation of corticosteroidogenic cells from head kidney of rainbow trout (Oncorhynchus mykiss) for testing cell-specific effects of a pesticide. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology* **147**, 52–60.

Jeffrey, J. D. and Gilmour, K. M. (2016). Programming of the hypothalamic-pituitary-interrenal axis by maternal social status in zebrafish (Danio rerio). *Journal of Experimental Biology* **219**, 1734–1743.

Jeffrey, J. D., Esbaugh, A. J., Vijayan, M. M. and Gilmour, K. M. (2012). Modulation of hypothalamic-pituitary-interrenal axis function by social status in rainbow trout. *Gen. Comp. Endocrinol.* **176**, 201–210.

Jeffrey, J. D., Gollock, M. J. and Gilmour, K. M. (2014). Social stress modulates the cortisol response to an acute stressor in rainbow trout (Oncorhynchus mykiss). *Gen. Comp. Endocrinol.* **196**, 8–16.

Johansen, I. B., Lunde, I. G., Røsjø, H., Christensen, G., Nilsson, G. E., Bakken, M. and Øverli, Ø. (2011). Cortisol response to stress is associated with myocardial remodeling in salmonid fishes. *Journal of Experimental Biology* **214**, 1313–1321.

Kageyama, K., Iwasaki, Y., Watanuki, Y., Niioka, K. and Daimon, M. (2021). Differential effects of fkbp4 and fkbp5 on regulation of the proopiomelanocortin gene in murine att-20 corticotroph cells. *Int. J. Mol. Sci.* **22**,.

Kubiak, M., Majewska, W., Kachel, M., Dola, A., Koga, W., Nowakowska, J., Langwiński, W. and Szczepankiewicz, A. (2025). Lithium Treatment Increases FKBP5 Protein but Not mRNA Expression in the Pituitary Gland of Depressive-like Rats. *Brain Sci.* **15**,.

Laidley, C. W. and Leatherland, J. F. (1988). Cohort sampling, anaesthesia and stocking-density effects on plasma cortisol, thyroid hormone, metabolite and ion levels in rainbow trout, Salmo gairdneri Richardson. *J. Fish Biol.* **33**, 73–88.

Li, Y., Liu, C., Bai, X., Li, M. and Duan, C. (2023). FK506-binding protein 5 regulates cell quiescence-proliferation decision in zebrafish epithelium. *FEBS Lett.* **597**, 1868–1879.

Liu, Y., Beyer, A. and Aebersold, R. (2016). On the Dependency of Cellular Protein Levels on mRNA Abundance. *Cell* **165**, 535–550.

Liu, N., Li, R., Cao, J., Song, X., Ma, W., Liu, T., Wang, L., Zou, J., Zhang, B., Liu, Z., et al. (2023). The inhibition of FKBP5 protects β-cell survival under inflammation stress via AKT/FOXO1 signaling. *Cell Death Discov.* **9**, 1–12.

McKibben, L. A., Iyer, M., Zhao, Y., Florea, R., Kuhl-Chimera, S., Deliwala, I., Pan, Y., Branham, E. M., Géranton, S. M., McLean, S. A., et al. (2025). Transcriptional changes across tissue and time provide molecular insights into a therapeutic window of opportunity following traumatic stress exposure. *Transl. Psychiatry* **15**, 1–13.

Mennigen, J. A., Magnan, J., Touma, K., Best, C., Culbert, B. M., Bernier, N. J. and Gilmour, K. M. (2022). Social status-dependent regulation and function of the somatotropic axis in juvenile rainbow trout. *Mol. Cell. Endocrinol.* **554**, 1–11.

Milewski, T. M., Lee, W., Champagne, F. A. and Curley, J. P. (2022). Behavioural and physiological plasticity in social hierarchies. *Philosophical Transactions of the Royal Society B: Biological Sciences* **377**, 1–14.

Montfort, J., Hervas-Sotomayor, F., Le Cam, A. and Murat, F. (2024). FEVER: An interactive web-based resource for evolutionary transcriptomics across fishes. *Nucleic Acids Res.* **52**, W65–W69.

Nagpal, J., Eachus, H., Lityagina, O. and Ryu, S. (2024). Optogenetic induction of chronic glucocorticoid exposure in early-life leads to blunted stress-response in larval zebrafish. *European Journal of Neuroscience* **59**, 3134–3146.

Øverli, Ø., Harris, C. A. and Winberg, S. (2000). Short-Term Effects of Fights for Social Dominance and the Establishment of Dominant-Subordinate Relationships on Brain Monoamines and Cortisol in Rainbow Trout. *Brain Behav. Evol.* **54**, 263–275.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, 2002–2007.

Piato, A. L., Capiotti, K. M., Tamborski, A. R., Oses, J. P., Barcellos, L. J. G., Bogo, M. R., Lara, D. R., Vianna, M. R. and Bonan, C. D. (2011). Unpredictable chronic stress model in zebrafish (Danio rerio): Behavioral and physiological responses. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **35**, 561–567.

Pickering, A. D. and Pottinger, T. G. (1989). Stress responses and disease resistance in salmonid fish: Effects of chronic elevation of plasma cortisol. *Fish Physiol. Biochem.* **7**, 253–258.

Pottinger, T. G. and Pickering, A. D. (1992). The influence of social interaction on the acclimation of rainbow trout, Oncorhynchus mykiss (Walbaum) to chronic stress. *J. Fish Biol.* **41**, 435–447.

Prodromou, C. (2016). REVIEW ARTICLE mechanisms of Hsp90 regulation. *Biochemical Journal* **473**, 2439–2452.

Rambo, C. L., Mocelin, R., Marcon, M., Villanova, D., Koakoski, G., de Abreu, M. S., Oliveira, T. A., Barcellos, L. J. G., Piato, A. L. and Bonan, C. D. (2017). Gender differences in aggression and cortisol levels in zebrafish subjected to unpredictable chronic stress. *Physiol. Behav.* **171**, 50–54.

Ramsay, J. M., Feist, G. W., Varga, Z. M., Westerfield, M., Kent, M. L. and Schreck, C. B. (2009). Whole-body cortisol response of zebrafish to acute net handling stress. *Aquaculture* **297**, 157–162.

Rein, T. (2016). FK506 binding protein 51 integrates pathways of adaptation: FKBP51 shapes the reactivity to environmental change. *BioEssays* **38**, 894–902.

Sadoul, B. and Vijayan, M. M. (2016). Stress and Growth. In *Fish Physiology*, pp. 167–205. Elsevier Inc.

Scharf, S. H., Liebl, C., Binder, E. B., Schmidt, M. V. and Müller, M. B. (2011). Expression and regulation of the Fkbp5 gene in the adult mouse brain. *PLoS One* **6**, 1–10.

Schreck, C. B. and Tort, L. (2016). The Concept of Stress in Fish. In *Fish Physiology*, pp. 1–34. Elsevier Inc.

Sloman, K. A., Metcalfe, N. B., Taylor, A. C. and Gilmour, K. M. (2001). Plasma cortisol concentrations before and after social stress in rainbow trout and brown trout. *Physiological and Biochemical Zoology* **74**, 383–389.

Talbot, J. C. and Amacher, S. L. (2014). A streamlined CRISPR pipeline to reliably generate zebrafish frameshifting alleles. *Zebrafish* **11**, 583–585.

Tao, H., Zhang, Y., Shen, Y., Chen, Q. and Liu, Z. (2025). Subacute temperature stress altered the fear response of male zebrafish by disrupting HPI/cortisol axis and NE/5-HT/DA neurotransmitter systems. *Environ. Biol. Fishes* **108**, 1099–1115.

Tea, J., Alderman, S. L. and Gilmour, K. M. (2019). Social stress increases plasma cortisol and reduces forebrain cell proliferation in subordinate male zebrafish (Danio rerio). *Journal of Experimental Biology* **222**, 1–10.

Teles, M., Tridico, R., Callol, A., Fierro-Castro, C. and Tort, L. (2013). Differential expression of the corticosteroid receptors GR1, GR2 and MR in rainbow trout organs with slow release cortisol implants. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology* **164**, 506–511.

Tokarz, J., Norton, W., Möller, G., Hrabé de Angelis, M. and Adamski, J. (2013). Zebrafish 20β-Hydroxysteroid Dehydrogenase Type 2 Is Important for Glucocorticoid Catabolism in Stress Response. *PLoS One* **8**, 1–14.

Tsachaki, M., Meyer, A., Weger, B., Kratschmar, D. V., Tokarz, J., Adamski, J., Belting, H. G., Affolter, M., Dickmeis, T. and Odermatt, A. (2017). Absence of 11-keto reduction of cortisone and 11-ketotestosterone in the model organism zebrafish. *Journal of Endocrinology* **232**, 323–335.

Tsoulia, T., Sundaram, A. Y. M., Amundsen, M. M., Aardal, M. J., Mira, M. S., Ploss, F. B., Faller, R., Jensen, I., Gjessing, M. C., Brauner, C., et al. (2025). Effects of glucocorticoid

receptor activation on gene expression and antiviral responses in Atlantic salmon (Salmo salar L.) red blood cells. *Vet. Res.* **56**,.

van Doeselaar, L., Stark, T., Mitra, S., Yang, H., Bordes, J., Stolwijk, L., Engelhardt, C., Kovarova, V., Narayan, S., Brix, L. M., et al. (2023). Sex-specific and opposed effects of FKBP51 in glutamatergic and GABAergic neurons: Implications for stress susceptibility and resilience. *Proc. Natl. Acad. Sci. U. S. A.* **120**,.

Vergauwen, L., Benoot, D., Blust, R. and Knapen, D. (2010). Long-term warm or cold acclimation elicits a specific transcriptional response and affects energy metabolism in zebrafish. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology* **157**, 149–157.

Wang, Y., Li, Z., Li, Z., Song, Y., Li, J., Yuan, L., Wang, C., Lai, F., Yan, R., Xiao, W., et al. (2025). Zebrafish fkbp5 attenuates antiviral innate immunity by autophagic degradation of transcription factor irf7. *Journal of Immunology* **214**, 1773–1788.

Winberg, S. and Lepage, O. (1998). Elevation of brain 5-HT activity, POMC expression, and plasma cortisol in socially subordinate rainbow trout. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **274**, 645–654.

Winberg, S. and Sneddon, L. (2022). Impact of intraspecific variation in teleost fishes: aggression, dominance status and stress physiology. *Journal of Experimental Biology* **225**, 1–11.

Wochnik, G. M., Rüegg, J., Abel, G. A., Schmidt, U., Holsboer, F. and Rein, T. (2005). FK506-binding proteins 51 and 52 differentially regulate dynein interaction and nuclear

translocation of the glucocorticoid receptor in mammalian cells. *Journal of Biological Chemistry* **280**, 4609–4616.

Wragg, J. and Müller, F. (2016). Transcriptional Regulation During Zygotic Genome Activation in Zebrafish and Other Anamniote Embryos. *Adv. Genet.* **95**, 161–194.

Yizhu, Z., Mando, Z., Aldurrah, Z., Mohamed Hanaphi, R., Abd Halim Hapiz, A. S., Azmi, M. A., Abdul Latip, A. S., Idris, S., Muhamad, A. and Zakaria, F. (2026). Metabolomic insights into gut–brain axis dysregulation under unpredictable chronic stress in zebrafish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **302**,.

Zannas, A. S., Wiechmann, T., Gassen, N. C. and Binder, E. B. (2016). Gene-Stress-Epigenetic Regulation of FKBP5: Clinical and Translational Implications. *Neuropsychopharmacology* **41**, 261–274.

Zimmer, C., Hanson, H. E. and Martin, L. B. (2021). FKBP5 expression is related to HPA flexibility and the capacity to cope with stressors in female and male house sparrows. *Horm. Behav.* **135**,.

Ziv, L., Muto, A., Schoonheim, P. J., Meijsing, S. H., Strasser, D., Ingraham, H. A., Schaaf, M. J. M., Yamamoto, K. R. and Baier, H. (2013). An affective disorder in zebrafish with mutation of the glucocorticoid receptor. *Mol. Psychiatry* **18**, 681–691.