The role of social and endocrinological context in regulating life history transitions among reproductive phenotypes in the bluebanded goby, Lythrypnus dalli

Devaleena Pradhan

Follow this and additional works at: https://scholarworks.gsu.edu/biology_diss

Recommended Citation
Pradhan, Devaleena, "The role of social and endocrinological context in regulating life history transitions among reproductive phenotypes in the bluebanded goby, Lythrypnus dalli." Dissertation, Georgia State University, 2014.
https://scholarworks.gsu.edu/biology_diss/144
THE ROLE OF SOCIAL AND ENDOCRINOLOGICAL CONTEXT IN REGULATING LIFE HISTORY TRANSITIONS AMONG REPRODUCTIVE PHENOTYPES IN THE BLUEBANDED GOBY, *LYTHRYPNUS DALLI*

by

DEVALEENA S PRADHAN

Under the Direction of Matthew S. Grober

ABSTRACT

During the lifetime of an organism, key events are orchestrated by a confluence of environmental, social, and physiological factors to promote reproductive success. Steroid hormones are critical regulators of fundamental aspects of reproductive life history, including gametogenesis, secondary sexual characteristics, sexual behavior, territory establishment and defense, and parenting. The steroid hormones investigated herein (testosterone (T), 11-ketotestosterone (KT), 17β-estradiol (E₂) and cortisol) are linked through steroidogenic conversion pathways. This dissertation utilized an integrative approach to investigate the neuroendocrine and social contexts that regulate transitions among phenotypes in a bidirectionally hermaphroditic haremic fish, *Lythrypnus dalli*. Conventional sex roles are reversed,
such that only males provide nest care, females exhibit intra-sexual competition and male reproductive success is associated with female courtship solicitation. Females living in stable social groups maintain dramatic differences in status, morphology, and tissue T, KT, E₂, and cortisol. Parasitic male morphs, mini males, do not defend territories and have morph-typical water-borne and tissue profiles of T, E₂, and KT. Two life history transitions, socially induced sex change and male parenting, are associated with increase in rates of behavior and KT levels. The regulation of these life history transitions by KT was investigated via two types of endocrine manipulations. Coupling systemic KT implants with a social context permissive to sex change caused rapid, but transient effects on agonistic behavior in dominant females, and secondary effects on subordinates during a period of social instability. Despite elevated brain and systemic KT 5 d after implant, overall rates of aggressive behavior remained unaffected, demonstrating a key role for context in regulating steroid associated changes in behavior. Intracerebroventricular inhibition of the enzyme 11β-hydroxysteroid dehydrogenase, reduced KT, elevated cortisol, and reduced male parenting behavior. 11-Ketotestosterone rapidly rescued parenting when administered along with the inhibitor, while cortisol had no effects on parenting. During reduced male nest attendance caused by KT inhibition, dominant, but not subordinate females, exhibited transient parenting and elevated brain KT. Taken together, rapid and/or local modulation of steroids allows for context-specific regulation of dynamic changes in behavior in an environment that requires an organism to successfully coordinate multiple activities to enhance fitness.

INDEX WORDS: Androgen, Challenge hypothesis, Cortisol, Courtship, Estradiol, Fitness, Parenting, Reproduction, Sex change, Social status, Testosterone
THE ROLE OF SOCIAL AND ENDOCRINOLOGICAL CONTEXTS IN REGULATING LIFE HISTORY TRANSITIONS AMONG REPRODUCTIVE PHENOTYPES IN THE BLUEBANDED GOBY, *LYTHRYPNUS DALLI*

by

DEVALEENA S PRADHAN

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the College of Arts and Sciences Georgia State University 2014
THE ROLE OF SOCIAL AND ENDOCRINOLOGICAL CONTEXT IN REGULATING LIFE HISTORY TRANSITIONS AMONG REPRODUCTIVE PHENOTYPES IN THE BLUEBANDED GOBY, *LYTHRYPNUS DALLI*

by

DEVALEENA S PRADHAN

Committee Chair: Matthew S. Grober

Committee: Laura L. Carruth
Andrew N. Clancy
Giovanni Gadda
Walter W. Walthall

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
August 2014
DEDICATION

To my parents, Ashok and Susmita, for their continual words of encouragement to follow my passions and pursue creativity and science.
ACKNOWLEDGEMENTS

I offer my gratitude to many people who have supported me throughout the duration of my doctoral studies. My advisor, Matthew Grober has always been available to provide me with unwavering guidance and encouraged me to think and work independently and express my opinions. He also taught me the importance of ‘process’, how to develop reasoning skills, and rational decision-making. Through his passion for the natural world, idealism, education, and invaluable friendship, he has motivated me when I have doubted myself.

The other members of my dissertation committee, Laura Carruth, Andrew Clancy, Giovanni Gadda, and Bill Walthall have always positively reinforced me, and offered stimulating discussions and guidance.

Without Tessa Solomon-Lane, my academic sister and good friend, my graduate experience would have been incomplete. She has always been there for me through difficult times, been a significant contributor to my research efforts, and kept a positive attitude.

Rosemary Knapp, Elizabeth Adkins-Regan, John Wingfield, John Godwin, Jacques Balthazart, Kim Wallen, Kiran Soma, Kim Schmidt, Scott MacDougall-Shakleton, Colin Saldanha, Thierry Charlier, and Chalotte Cornil have played significant roles in my scientific development through discussions at conferences, suggestions on experiment design, and reviews.

This dissertation would not be possible without the support of several present and past members of Matthew Grober’s laboratory, who have helped me throughout these years, including Kim Connor, Cory Grober, Madie Willis, Kevin Thonkulpitak, Eric Schuppe, Hannah Shin, Elizabeth Pritchett, Pierre Naude, Jason Crutcher, Ravi Batra, Michael Black, Varenka Lorenzi, Ed Rodgers, Caitlin McCoyd, Megan Williams, Captain Jack, David Sinkiewicz, Joseph Bush, and Polina Shvidkaya.
I thank Charles Derby, Anne Murphy, Kyle Frantz, Marise Parent, Aaron Roseberry, Vincent Rehder, Lock Rogers, Matt Paul, Mario Gil, Rob Clewley, Neil van Leeuwen, and Matthew Nusnbaum for years of support, questions, discussions, and for their valued friendships.

The following staff and research personnel at GSU were integral to the successful completion of my dissertation: Mary Karom, Siming Wang, Lifang Wang, LaTesha Warren, Debbie Walthall, Rob Poh, Anwar Lopez, Fatima Adams, Tamara Gross, Lindsey Hornsby, Barry Grant, Karon Mackey, and Tara Alexander. Liz Weaver has been instrumental for enhancing my experience at GSU through all the excursions she organized through the Brains & Behavior Program, cheered me all the way, and has been a great friend.

Kellie Spafford, Lauren Czarnecki Oudin, Trevor Oudin, Josh Rinker, Kory Gozjack, Gordon Boivin, and other members of the staff at Wrigley Center for Environmental Studies who not only helped make my research endeavors successful, but have also become lasting friends.

Swathi Gannavaram, Tim Balmer, Charuni Gunnaratne, Mahin Shahbazi, Melissa Bucheit, Stephen Estes, Sushma Reddy, Amy Ross, Nicole Victoria, Jenna Darling, Ray Zhong, Laura Been, Emily Bruggeman, Marc Badura, Arpana Sagwal, Johnny Garreston, and Bryce Chung, have all contributed to my personal growth and experience at GSU in unique ways.

Debi Grober, Seema Koner, Raj Koner, Joyeeta Chatterjee, Sanjana Aswani, Neera Sharma, Sharon Sen, Aparna Giridharadas, Nicole Hofs, and Anjana Moitra are my friends outside GSU who brought a different perspective and sincere emotional support to my life and treated me like family.

Above all, I thank my parents, Ashok and Susmita Pradhan and the rest of my family for always being with me through their words of confidence and inspiration despite the long distance.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS .................................................................................................................... v

LIST OF TABLES ................................................................................................................................. xv

LIST OF FIGURES ................................................................................................................................. xvi

LIST OF ABBREVIATIONS .................................................................................................................... Error! Bookmark not defined.

1 INTRODUCTION ................................................................................................................................. 1

1.1 Context of physical environment ................................................................................................. 2

1.1.1 Physical environmental context in Lythrypnus dalli .............................................................. 3

1.2 Social context ................................................................................................................................ 3

1.2.1 Social context in Lythrypnus dalli ......................................................................................... 5

1.3 Endocrine context ............................................................................................................................ 6

1.4 Proxies of steroid function ............................................................................................................. 7

1.5 Endocrine manipulations ............................................................................................................... 11

1.5.1 Peripheral manipulations .......................................................................................................... 12

1.5.2 Local manipulations ................................................................................................................ 13

1.5.3 Limitations ................................................................................................................................ 13

1.5.4 Endocrine context in Lythrypnus dalli ................................................................................... 14

1.6 References ....................................................................................................................................... 15

2 CONTEXTUAL MODULATION OF ANDROGEN EFFECTS ON AGONISTIC INTERACTIONS ................................................................................................................................................ 28
2.1 Abstract .......................................................................................................................... 28

2.2 Introduction ................................................................................................................... 29

2.3 Methods .......................................................................................................................... 33

2.3.1 Subjects ....................................................................................................................... 33

2.3.2 General procedures ..................................................................................................... 34

2.3.3 Steroid manipulations ................................................................................................. 35

2.3.4 Behavioral observations ............................................................................................. 36

2.3.5 Hormone assays .......................................................................................................... 37

2.3.6 Statistical analyses ..................................................................................................... 38

2.4 Results ............................................................................................................................ 40

2.4.1 Effect of male removal and hormone manipulation on morphology .................. 40

2.4.2 Effect of hormone manipulations on physiology ..................................................... 41

2.4.3 Effect of hormone manipulation on behavior ............................................................ 41

2.4.4 Agonistic efficiency .................................................................................................... 43

2.5 Discussion ....................................................................................................................... 44

2.5.1 Hormone measurements as physiological proxies ...................................................... 45

2.5.2 Rapid and context-specific behavioral effects ............................................................. 47

2.5.3 Alpha behavior may cause changes in beta behavior ................................................. 50

2.5.4 No long-term effects on rate of aggressive behavior ................................................. 52

2.5.5 Conclusions ............................................................................................................... 53
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.6</td>
<td>Acknowledgements</td>
<td>53</td>
</tr>
<tr>
<td>2.7</td>
<td>References</td>
<td>54</td>
</tr>
<tr>
<td>3</td>
<td>WATER-BORNE AND TISSUE ENDOCRINE PROFILES OF AN ALTERNATIVE REPRODUCTIVE PHENOTYPE IN THE SEX CHANGING FISH, <em>LYTHRYPNUS DALLI</em></td>
<td>70</td>
</tr>
<tr>
<td>3.1</td>
<td>Abstract</td>
<td>70</td>
</tr>
<tr>
<td>3.2</td>
<td>Introduction</td>
<td>71</td>
</tr>
<tr>
<td>3.3</td>
<td>Methods</td>
<td>75</td>
</tr>
<tr>
<td>3.3.1</td>
<td>Field protocol</td>
<td>75</td>
</tr>
<tr>
<td>3.3.2</td>
<td>Steroid collection, extraction, and enzyme immunoassays</td>
<td>76</td>
</tr>
<tr>
<td>3.3.3</td>
<td>Statistical analyses</td>
<td>79</td>
</tr>
<tr>
<td>3.4</td>
<td>Results</td>
<td>80</td>
</tr>
<tr>
<td>3.4.1</td>
<td>Water-borne steroids</td>
<td>80</td>
</tr>
<tr>
<td>3.4.2</td>
<td>Tissue steroids</td>
<td>80</td>
</tr>
<tr>
<td>3.4.3</td>
<td>Regression analyses</td>
<td>81</td>
</tr>
<tr>
<td>3.4.4</td>
<td>Relationship between steroids</td>
<td>81</td>
</tr>
<tr>
<td>3.5</td>
<td>Discussion</td>
<td>81</td>
</tr>
<tr>
<td>3.5.1</td>
<td>Water-borne steroids</td>
<td>82</td>
</tr>
<tr>
<td>3.5.2</td>
<td>Tissue steroids</td>
<td>83</td>
</tr>
<tr>
<td>3.5.3</td>
<td>Water-borne versus tissue steroids</td>
<td>85</td>
</tr>
</tbody>
</table>
4 A MECHANISM FOR RAPID NEUROSTEROIDAL REGULATION OF PARENTING BEHAVIOR

4.1 Abstract

4.2 Introduction

4.3 Methods

4.3.1 General methods

4.3.2 Experiment 1: effect of CBX on in vitro 11β-HSD activity

4.3.3 Experiment 2: Effect of systemic CBX implants on KT exuded into water

4.3.4 Experiment 3: effect of ICV CBX on male parenting

4.3.5 Experiment 4: effect of context on male reproductive success, female behavior, and tissue KT

4.4 Results

4.4.1 Experiment 1: effect of CBX on in vitro 11β-HSD activity

4.4.2 Experiment 2: effect of CBX implants on KT exuded in water
4.4.3  Experiment 3: effect of ICV CBX on male parenting.......................... 117

4.4.4  Experiment 4: effect of context on male reproductive success, female

behavior, and tissue KT........................................................................................................ 119

4.5  Discussion.......................................................................................................................... 119

4.5.1  Effect of CBX on steroid hormone conversion pathways ......................... 120

4.5.2  Neurosteroidal regulation of parenting behavior............................................ 121

4.5.3  Role of neural steroidogenic enzymes in regulating transitions between

behavioral states .................................................................................................................... 123

4.5.4  Conclusions.................................................................................................................. 124

4.6  Acknowledgements.......................................................................................................... 125

4.7  References........................................................................................................................ 125

5  MULTI-TASKING MALES AND SEX-ROLE REVERSED FEMALES IN

HAREMIC BLUEBANDED GOBIES, LYTHRYPNUS DALLI.......................................... 139

5.1  Abstract............................................................................................................................. 139

5.2  Introduction....................................................................................................................... 140

5.3  Methods............................................................................................................................. 143

5.3.1  General procedures..................................................................................................... 143

5.3.2  Study 1......................................................................................................................... 143

5.3.3  Study 2......................................................................................................................... 144

5.4  Results............................................................................................................................... 148
5.4.1  Study 1........................................................................................................ 148

5.4.2  Study 2........................................................................................................ 149

5.5  Discussion......................................................................................................... 151

5.5.1  Egg presence had no effect on rates of courtship behavior.................... 152

5.5.2  Social interactions might limit male parenting................................. 153

5.5.3  Female biased sex ratio permits intra-female competition and sex role
        reversal ........................................................................................................ 156

5.6  Acknowledgements......................................................................................... 157

5.7  References........................................................................................................ 157

6  TISSUE STEROID LEVELS ARE ASSOCIATED WITH FEMALE SOCIAL
       STATUS, BUT NOT RATES OF BEHAVIOR NOR OVARIAN FUNCTION............ 172

6.1  Abstract........................................................................................................... 172

6.2  Introduction....................................................................................................... 173

6.3  Methods............................................................................................................ 177

   6.3.1  Subjects..................................................................................................... 177

   6.3.2  General procedures.................................................................................. 178

   6.3.3  Morphological measurements............................................................... 178

   6.3.4  Behavior measures and social contexts................................................. 179

   6.3.5  Steroid hormone assays.......................................................................... 180

   6.3.6  Statistical analyses.................................................................................... 181
6.4 Results ........................................................................................................ 182

6.4.1 Morphology .............................................................................................. 182

6.4.2 Behavior .................................................................................................... 183

6.4.3 Ratios ......................................................................................................... 185

6.5 Discussion ..................................................................................................... 185

6.5.1 Morphology does not influence status specific steroid hormone levels.... 186

6.5.2 Regardless of context, females maintain status specific steroid hormone
styles .............................................................................................................. 187

6.5.3 Tissue hormones indicate status ............................................................... 188

6.5.4 Interaction among steroid hormones ....................................................... 190

6.5.5 Conclusions .............................................................................................. 192

6.6 Acknowledgements ...................................................................................... 192

6.7 References .................................................................................................... 193

7 DISCUSSION ................................................................................................... 210

7.1 Breaking biological boundaries: core endocrinological concepts being
addressed ........................................................................................................... 210

7.2 Organization and activation of phenotype ................................................. 212

7.3 Regulation of phenotype ............................................................................. 213

7.4 Brain steroid synthesis regulates behavior ................................................. 214

7.5 Steroids have rapid effects on behavior ..................................................... 215
7.6 Androgens do not increase aggressive behavior unless under specific contexts 216

7.7 Androgens and glucocorticoids are not negatively associated with parenting 217

7.8 Behavior of one individual affects other members of the social group .... 218

7.9 Fitness proxies help determine the consequences of neuroendocrine regulation of behavior ................................................................. 219

7.10 Future directions ........................................................................................................ 219

7.11 References .................................................................................................................. 221
LIST OF TABLES

Table 3.1: Relationship between water-borne and tissue sex steroids in *L. dalli* mini males. .................................................................................................................................................. 94

Table 3.2: No significant relationship between sex steroids and standard length in *L. dalli* mini males........................................................................................................................................................................ 95

Table 3.3: Relationship between tissue steroid levels and genital papilla length to width ratio in mini males of *L. dalli*. .................................................................................................................................................. 96

Table 5.1: Relationship between total number of eggs (orange + eyed) and behavior of *L. dalli* ........................................................................................................................................................................ 163

Table 5.2: Relationship between number of eyed eggs and behavior of *L. dalli*........ 164

Table 6.1: Morphological Differences between alpha females and beta females in *L. dalli* ........................................................................................................................................................................ 199

Table 6.2: Effects of male behavior and female status on tissue steroid hormones. Results of within subjects two-way ANOVA are presented. .................................................................................................................................................. 200

Table 6.3: Effects of alpha female behavior and female status on tissue steroid hormones. ........................................................................................................................................................................ 201

Table 6.4: Comparison of relative tissue steroid hormone ratios in female *L. dalli*...... 202
LIST OF FIGURES

Figure 1.1: Social context in Lythrypnus dalli. (A) Individuals in a stable social group, form a linear social hierarchy, in which the male is the most dominant member and females are in a social environment that is inhibitory to sex change. (B) Upon male removal, individuals are in a transitioning social group, such that the alpha female is in a social environment that is permissive to sex change and the more subordinate females are in a social environment that is inhibitory to sex change. The Dominance Phase is a transitional period where status resolution takes place, after which the social hierarchies return to stability. .......................................................... 26

Figure 1.2: Proxies of steroid function. There are many different approaches to determine steroid levels in organisms.......................................................... 27

Figure 2.1: Genital papillae (GP) structure in L. dalli (A) three representative L. dalli transitioning females treated with Chol or KT on d0, d3, and d5; location of the vent is shown by the arrow, and the measurements of the length (L) and width (W) are indicated (B) a representative male GP (C) effect of implants on GP length to width ratio against time relative to steroid treatment. (n=10 Chol and n=11 KT). *p<0.05. .................................................................................. 61

Figure 2.2: 11-ketotestosterone implant pellets. (A) Relationship between steroid implant weight (Chol and KT) and standard length of alpha females (B) Pre-implant, initial (d0) and post implant, final (d5) Chol and KT implant. .................................................................................. 62

Figure 2.3: Levels of KT in L. dalli before (females) and after 5 d (transitioning males) of treatment with Chol or KT in (A) Systemic (water-borne): pre-implant is d0, post-implant is d5 and (B) Local (tissue) levels in brain, gonad and muscle of transitioning L. dalli on d5 (n=10 Chol and n=11 KT). **p<0.01, ***p<0.001. .................................................................................. 63
Figure 2.4: Relationship between the amounts of KT absorbed by KT implanted alphas (initial – final weights of KT pills) and the level of KT in the gonad. Amount of KT absorbed negatively correlated with gonadal KT levels. (n=10 Chol and n=11 KT). ........................................ 64

Figure 2.5: Rates of agonistic interactions between alpha and beta L. dalli relative to the time before or after alphas were treated with either KT or Chol (A) Alpha approaches beta (B) Alpha displaces beta (C) Beta approaches alpha, and (D) Beta displaces alpha. * p<0.05. (n=8 Chol and n=10 KT) Dotted lines represent the transient window of social instability that follows male removal (“the dominance phase” (Reavis and Grober, 1999)). ........................................... 65

Figure 2.6: Relationships between rates of agonistic behavior of alphas treated with Chol or KT; (A) Behavior and systemic KT on d0 (B) d4 behavior and systemic KT on d5, and (C) d4 behavior and brain KT on d5. ............................................................................................................. 66

Figure 2.7: Total time spent in the nest by (A) Alphas and (B) Betas relative to time after implant. (n=10 Chol and n=11 KT); ***p< 0.001 ............................................................................................................. 67

Figure 2.8: Agonistic efficiency of alpha and beta females. (A) Alpha agonistic efficiency (AgEf, displacements/approaches) towards betas relative to time after implant, (B) Beta AgEf towards alphas relative to time after implant (n=8 Chol and n=10 KT)......................... 68

Figure 2.9: Categorical representation of rates of beta approaches when alpha AgEf was < or > 0.5. The numbers above bars represent the number of groups for each category. The inset is a regression of alpha AgEf against rates of betas approaching alphas after alphas were treated with either KT or Chol (N=15). Note that some individuals were excluded from this analysis due to zero rates of interaction and overlap does not allow all data points to be seen clearly. .......... 69
Figure 3.1: Water-borne (A) and tissue (B) levels of sex steroids (mean ± S.E.M) in mini males (n=9). Repro., reproductive tissue (testes + AGS). Different letters above bars indicate that the means are significantly different from each other; *p<0.05, **p<0.01......................... 97

Figure 3.2: Linear regressions between water-borne (A) testosterone, (B) 17β-estradiol and (C) 11-ketotestosterone levels and genital papilla morphology (expressed as Length:Width ratio) in L. dalli mini males. (n=9). Averages reported in (Lorenzi et al., 2012) were used to estimate ratios for females and parenting males. T, testosterone; E\textsubscript{2}, 17β-estradiol; KT, 11-ketotestosterone. ........................................................................................................... 98

Figure 3.3: Relative tissue sex steroid ratios from (A) brain (B) reproductive tissue, and (C) muscle of Lythrypnus dalli parenting males, females and mini males. For mini males, mean ± SEM is reported. ..................................................................................................................................... 99

Figure 3.4: Relative systemic sex steroid ratios from four species of Teleost fish (A) Porichthys notatus, plainfish midshipman, (B) Parablennius sanguniolentus parvicornis, rock-pool blenny, (C) Lepomis macrochirus, bluegill sunfish, and (D) Lythrypnus dalli, bluebanded goby. Averages reported in (Lorenzi et al., 2008; Knapp and Neff, 2007; Oliveira et al., 2001; Brantley et al., 1993; Sisneros et al., 2004) were used to estimate ratios. Data for KT are not available for females of some species. T, testosterone; E\textsubscript{2}, 17β-estradiol; KT, 11-Ketotestosterone, N.D., non-detectable. ............................................................................................................. 100

Figure 4.1: Simplified pathway of steroidogenesis in fish. Testosterone is converted to 11-Ketotestosterone (KT) via the sequential action of 11β-hydroxylase, which converts KT to 11β-hydroxytestosterone (11β-OHT), and 11β-hydroxysteroid dehydrogenase, which converts 11β-OHT to KT and cortisol to cortisone. Inhibition of 11β-HSD with carbenoxolone (solid grey ‘X’) can elevate cortisol and reduce KT (solid grey arrows) levels. ................................................................. 132
Figure 4.2: Effect of carbenoxolone (CBX) and 11β-hydroxytestosterone (OHT, an endogenous substrate) on 11β-hydroxysteroid dehydrogenase activity in adult male L. dalli (a) brain and (b) testes. Tissue supernatants were incubated *in vitro* at 25°C with 1 mM NAD⁺ as a co-substrate for 60 min. Controls were incubated with buffer + vehicle only. 11-Ketotestosterone (KT) peak area ratio was calculated by dividing area of KT peak to that of corticosterone (B), the internal LCMS standard (N=3 males per group); **p<0.01, ***p<0.001.

Figure 4.3: Effect of carbenoxolone (CBX) and 11β-hydroxytestosterone (OHT, an endogenous substrate) on 11β-hydroxysteroid dehydrogenase activity in adult male L. dalli (a) brain and (b) testes. Tissue supernatants were incubated *in vitro* at 25°C with 1 mM NAD⁺ as a co-substrate for 60 min. Controls were incubated with buffer + vehicle only. 11-Ketotestosterone (KT) peak area ratio was calculated by dividing area of KT peak to that of corticosterone (B), the internal LCMS standard (N=3 males per group); **p<0.01, ***p<0.001.

Figure 4.4: Effect of intraperitoneal (IP) implants of carbenoxolone (CBX), an 11β-hydroxysteroid dehydrogenase inhibitor, on 11-ketotestosterone (KT) exuded in water (systemic levels) 1 h, 1 d, and 4 d post-treatment of adult male L. dalli. Vehicle: n=5 and CBX: n=4; *p<0.05, **p<0.01

Figure 4.5: Effect of intracerebroventricular (ICV) injection of parenting male L. dalli on (a) latency to enter nest, (b) time spent in nest between 10-20 min, and (c) number of parenting bouts between 10-20 min. Vehicle: n=7; Carbenoxolone (CBX): n=9; CBX + 11-ketotestosterone (KT): n=9; Cortisol: n=9; *p<0.05, **p<0.01
Figure 4.6: Effect of intracerebroventricular (ICV) injection of parenting male *L. dalli* on agonistic interactions and parenting behavior; (a) male approach rate, (b) male displacement rate, and (c) male parenting. Vehicle: n=7; Carbenoxolone (CBX): n=9; CBX + 11-ketotestosterone (KT): n=9; Cortisol: n=9; *p<0.05 **p<0.01 .............................. 137

Figure 4.7: Effect of ICV treatment of parenting males on female interaction with eggs (a) Percentage of egg loss in groups with males treated with vehicle (n=7) or CBX (n=9) (b) Female egg eating bouts as a function of time relative to ICV injections of the male (c) Brain and (d) Ovarian KT levels in females who did not parent (n=22) and those who exhibited parenting (n=12); *p<0.05, **p<0.01 .............................. 138

Figure 5.1: An illustration showing scenarios and rules for scoring courtship solicitation postures by *L. dalli* females (top views). The female must align her body perpendicular to the male so as to display the state of her distended abdomen (gravidity) to the male, the female must remain stationary and not engaged in interaction with another individual or feeding. (A) The female could be positioned anterior or (B) posterior to the male (C) Some part of the female body must be intersected by the median linear axis of the male. ................................. 165

Figure 5.2: groups of *L. dalli* show within and between group natural variation in (A) Average total number of eggs (orange + eyed) laid on 8 different days (B) Average number of nest care (fanning and rubbing) bouts exhibited on 9 different days during 10 min observation sessions, over the course of 3 weeks in social groups housed in a semi-natural laboratory environment. Each social group (N=16) consisted of one male and two size-mismatched females. Error bars for each group captures variation within groups and across time. .......... 166

Figure 5.3: Effect of egg presence on rates of *L. dalli* male behavior (A) male approaches towards females (B) male parenting over a 3 week period. Each group comprised of one male
and two status mismatched females. Eight behavioral observations were conducted for N=16 groups.

Figure 5.4: Effect of egg presence on rates of courtship behavior exhibited by *L. dalli* living in groups comprised of one male (M) and two status mismatched females (α= alpha female, β= beta female) during egg absence and presence over a 3 week period. When there are no eggs in the nest, rates of beta solicitations are higher than males jerk rate. Eight behavioral observations were conducted for N=16 groups. The letters inside the histogram represent sex-specific courtship behavior, J= male jerks, S= female solicitation, *p<0.05.*

Figure 5.5: Relationship between total number of eggs (orange + eyed) and rates of *L. dalli* male behavior (A) proportion of time spent parenting (fanning and rubbing) (B) male courtship jerks (C) male approaches towards females. Nine behavioral observations were conducted for N=16 groups over a 3 week period.

Figure 5.6: Relationship between number of eggs and rates of *L. dalli* female behavior (A) alpha + beta solicitation and total number of eggs (B) alpha solicitation and number of eyed eggs. Nine behavioral observations were conducted for N=16 groups over a 3 week period.

Figure 5.7: Simplified flow chart showing the pattern of interactions within *L. dalli* groups consisting of one male, an alpha female, and a beta female, based on first order Markov transitions. A total of 875 transitions were used to generate the chart, and arrow thickness is proportional to the frequency (converted into percentage, and noted beside the arrow) of the transition. To reduce complexity, only frequencies >0.1 (10%) are shown.

Figure 6.1: Representative photographs of ovarian function and changes in egg morphology during the breeding season in female *L. dalli* collected off the coast of Santa Catalina Island. Ovarian scores are assigned by stage in ovarian cycle.
Figure 6.2: Frequency distribution of dominant and subordinate female *L. dalli* based on (A) ovarian score and (B) gonadosomatic index. Females were living in stable social groups consisting one male and two size- and status-mismatched females for 4 weeks. Alpha females: n=34 and beta females: n=34.

Figure 6.3: Levels of 17β-estradiol in ovarian tissue categorized by stages of ovarian development (ovarian score) of all alpha and beta female *L. dalli*; n=68. The number inside each bar denotes the sample size of each group. **p<0.01, *p<0.05.

Figure 6.4: Effects of treating male *L. dalli* on baseline and post-treatment (A) agonistic efficiency and (B) duration in nest(s) of all individuals in stable *L. dalli* groups consisting of one male and two status- and size-mismatched females. Males: n=34; Alpha females: n=34; Beta females; ***p<0.001.

Figure 6.5: Levels of brain (A) Testosterone (B) 11-Ketotestosterone (C) 17β-estradiol and (D) Cortisol of female *L. dalli* living in stable social groups consisting one male and two size- and status-mismatched females for 4 weeks. Alpha females (dominant): n=34 and beta females (subordinate): n=34. Alpha females: n=34; Beta females: n=34. Note that the y-axis is different for all hormones. **p<0.01, ***p<0.001.

Figure 6.6: Levels of ovarian (A) Testosterone (B) 11-Ketotestosterone (C) 17β-estradiol and (D) Cortisol of female *L. dalli* living in stable social groups consisting one male and two size- and status-mismatched females for 4 weeks. Alpha females (dominant): n=34; Beta females (subordinate): n=34. Note that the y-axis is different for all hormones. *p<0.05.

Figure 6.7: Comparison of relative tissue hormone ratios (A) Brain and (B) Ovary of dominant and subordinate female *L. dalli*. Alpha females: n=34; Beta females: n=34. **p<0.01, **p<0.001.
Figure 7.1: Functional interpretation of endocrine studies should be cognizant of the relevant proxy. Loose links exist, such that expression of phenotype is dependent upon both, (A) endocrine and (B) social factors. (C) Balance between these two factors is necessary for the regulation of function and increase lifetime reproductive success. (D) Several endocrine and social context factors must be considered when designing experiments.
1 INTRODUCTION

Most organisms exhibit distinct developmental and reproductive stages during their life cycle. Physiological factors are critical to orchestrate transitions between those stages and coordinate dynamic context-specific activities to increase fitness. Within this realm, steroid hormones regulate phenotypes such as behavior and morphology in response to environmental change (Remage-Healey and Romero, 2000; Wingfield et al., 1990a). Long-term steroid effects are most often mediated via classical genomic processes, while short-term, rapid effects can be mediated via non-genomic mechanisms. Circulating steroids, presumed to be of gonadal origin, are often measured to understand endocrine regulation of behavior (Borg, 1994a; Magee et al., 2006). For example, in seasonally breeding animals, there are marked breeding season increases in gonadal androgen synthesis (associated with spermatogenesis), positively correlated with circulating androgens and sexual and territorial behavior (Borg, 1994a; Wingfield et al., 1990a). Intriguingly, some species maintain territorial aggression outside the breeding season despite low circulating androgen levels (Caldwell et al., 1984; Soma, 2006). One mechanism by which aggression during the non-breeding season can be maintained is through brain synthesis of androgens (Pradhan et al., 2010b). The overall goal of this dissertation is to improve our understanding of mechanisms involved in regulating phenotypes by manipulating steroids in vivo, investigating different levels of analyses within the context of proxies of endocrine measurement.

This dissertation will address the roles of four different steroid hormones, testosterone (T), 11-ketotestosterone (KT), 17β-estradiol (E₂) and cortisol during life history transitions of the bi-directionally, hermaphroditic marine fish, *Lythrypnus dalli*. To pursue the goal, I will address the following questions: (1) Does elevation of systemic KT affect tissue KT levels and
phenotype during sex change?  
(2) What are the water-borne and tissue endocrine (T, KT, E₂) correlates of mini males?  
(3) Does inhibition of the enzyme 11β-hydroxysteroid dehydrogenase affect brain KT and cortisol levels and phenotype during parenting?  
(4) What are the reproductive parameters and behavioral phenotypes in stable social groups of *L. dalli*?  
(5) What are the morphological, behavioral, and hormonal (T, KT, E₂, and cortisol) differences between dominant and subordinate females living in stable social groups?  

To answer these questions, I will use an integrative approach including:  
1) *in vitro* biochemical studies  
2) *in vivo* systemic (implants) and local intracerebroventricular (ICV injections) endocrine manipulations;  
3) systemic and local endocrine measurements;  
4) anatomical measurements; and  
5) reproductive success measures, all done within the context of  
6) in-depth behavioral observations of wild-caught fish in a semi-natural laboratory setting.  

Below, I briefly discuss the context of environment, but focus on my proposed concept of endocrine context.

### 1.1 Context of physical environment

The physical environment can cause both predictable and unpredictable changes in endogenous hormones, either at the level of synthesis or signaling cascades for downstream consequences (Pradhan and Soma, 2012). All activities of an organism generally focus around nutrition and reproduction, and in turn, both of these activities are broadly dependent upon temporal patterns associated with circannual and seasonal rhythms (Ball et al., 2004; Prendergast et al., 2009). The geographic location can serve as a selective pressure regulating physiological mechanisms (Nelson, 2011; Wingfield, 2012). For example, the patterns of genotype, phenotype, and plasticity of expression are rather different for animals living in the Tropics, Temperate, and Arctic regions (Borg, 1994b; Wingfield et al., 1990b; Wingfield and Hunt,
2002). Additionally, the aspect of natural versus the laboratory environment critical to consider; for example, natural populations have high hormone levels and natural predator stress can cause a greater glucocorticoid response than what can be simulated via restraint (Newman et al., 2013). It is imperative that sex steroids and glucocorticoids have differential responses to environmental stressors (Narayan et al., 2012), such that the tradeoffs favor an instinct for survival, rather than reproduction. Hence the specific endocrine response studied must be based on the appropriate environmental context.

1.1.1 Physical environmental context in *Lythrypnus dalli*

All the experiments in this dissertation were performed on a species of marine fish dwelling in the temperate waters off the shore of Santa Catalina Island (33.44°N 118.49°W). The habitat of *L. dalli* ranges from the benthic rocky reefs of the Gulf of California, Mexico to Morro Bay, California (Miller and Lea, 1972). Free-living *L. dalli* live in mixed sex groups in association with the crowned urchin, *Centrostephanus coronatus*. All fish were captured during the breeding season in the months of June – August between 2009 and 2012. They were brought either to the laboratory at Wrigley Center for Environmental Studies and studied there during the summer months or shipped to Georgia State University.

1.2 Social context

In the wild, an organism might experience variation in the type of social interaction with conspecifics throughout its lifetime. Endogenous steroids regulate social interactions in a context dependent manner. To control for environmental variation and logistical constraints of laboratory-based experiments, most studies use a very simplified and/or artificial social environment. However, in group-living species, social complexity interplays with hormones to
impact reproductive behavior. For example, in a multi-female groups of rhesus monkeys, *Macaca mulatta*, males direct their reproductive behavior towards females only during the peri-ovulatory phase (Wallen and Winston, 1984). In pair-housing, however, males direct reproductive behavior towards females during both the follicular and peri-ovulatory phase (Wallen and Winston, 1984). However, females are receptive to copulation only during particular reproductive stages in rhesus monkeys (Wallen and Winston, 1984) and also in other vertebrates such as rodents (Kow et al., 1978) and amphibians (Lynch et al., 2006). Thus, parameters are shaped by cyclical ovarian patterns, which dictate the expression of specific reproductive behaviors in both males and females.

Traditionally, hormone manipulations have been used to understand the role of steroids in the proximate regulation of behavior. When hormones are manipulated to determine the mechanisms by which they regulate behavior, the probability of increasing or decreasing the expression of those behaviors (e.g. aggressive, sexual) is complicated because these behaviors are often exhibited in tightly regulated windows and dependent on the integration of several ecological, social, and endogenous factors. For example, treatment of spotted antbirds, *Hylophylax n. naevioides* with pharmacological inhibitors of androgen and estrogen actions are not as effective in non-breeding season compared to the breeding season and also dependent on the intensity of aggressive stimuli (Beebee, 2004). Experimentally elevated hormones often have impacts only during a short period after the manipulation and only specific components of aggression could be affected. In black redstarts, *Phoenicurus ochruros*, T levels reduce in the short-term and this affects only specific components of song structure (Apfelbeck et al., 2013). Moreover, these effects also depend on whether the behavior is directed in a conspecific aggression or mating context (Apfelbeck et al., 2013). Thus timing and social group dynamics
are both important factors when determining the proximate hormonal mechanisms regulating behavior.

1.2.1 Social context in *Lythrypnus dalli*

The bluebanded goby is a bi-directionally hermaphroditic fish (Reavis and Grober, 1999) that develops through a series of distinct life history stages. The social behavior and ecology of this species is well characterized in both the laboratory and field, and there are a variety of methods and data that serve as a strong foundation for further investigation of neuroendocrine mechanisms of life history transitions. Social groups are easily set up under conditions that are *permissive* for natural sex change and for spawning (Figure 1.1). The male, the most dominant member of the social group, is provided with a harem of 2-3 size-mismatched females and a piece of PVC tube that serves as his nest. These groups normally establish a robust linear hierarchy within 5 d (Reavis and Grober, 1999). Male courtship behavior involves jerk swims towards females of his choice, whom he leads into the nest for spawning. Very little is known about female courtship solicitation and how it impacts reproductive success. Demersal eggs are exuded through the genital papilla of the female and attached to the substrate with adhesive filaments. The male paints sperm over the eggs and cares for them until hatching. Paternal care includes aerating eggs with fanning and rubbing behaviors and displaying aggressively towards females and other predators (in the wild) who may consume eggs. These eggs can be visually inspected to determine whether they are newly laid (orange), late embryos (‘eyed’ eggs), or yolk-sac larva (transparent, with more prominent eye pigmentation). Upon hatching, the pelagic larvae go through a planktonic phase, after which they recruit onto the reef and undergo metamorphosis into juveniles (12-17 mm range SL). All experiments in this dissertation were performed on adults.
In the presence of the male, all females are in an environment inhibitory to sex change (Figure 1.1). Sex change can then be readily induced in the alpha female by removing the male. Immediately following male removal (first few minutes), the rates of agonistic interactions among the females increase while they re-establish social status relationships (Reavis and Grober, 1999). The highest-ranking female, usually the most aggressive, is not subordinated by any other female (Rodgers et al., 2007), and rapidly assumes the dominant and thus male position. The most dominant female is now in a transitioning social environment that is permissive for sex change, while the subordinate females are in an environment inhibitory for sex change. Depending upon the specific group, there is substantial variation in the time over which a stable social hierarchy is re-established (“Dominance Phase”), after which there is a decline in rates of agonistic interactions (Reavis and Grober, 1999). I took these aspects of the *L. dalli* social paradigm into consideration when designing experiments.

### 1.3 Endocrine context

Steroid hormones mitigate environmental signals to transcend information to behavioral command centers (Alcock, 2001). However, these signals can be detected only when the endogenous state of the organism is primed to sense the exogenous (social and physical) environment within which it lives. This signal transduction occurs via cellular and molecular mechanisms and can be misrepresented or misinterpreted unless considered within the context of location of responses to finally have a phenotypic effect (Ball and Balthazart, 2008). Based on the organism studied, there are several different types of biological samples that could serve as proxies of steroid bioavailability. To interpret the mechanism by which steroids function and have their activating effects, several techniques of steroid manipulation could be used. To
interpret the importance of steroids to maintain normal function, all levels of endocrine context should be considered within the scope of life history transitions of organisms.

1.4 Proxies of steroid function

Collectively, steroids affect several facets of phenotype and each steroid can have multiple effects (Nelson, 2011). These steroid functions include, and are not limited to, production of gametes, activities related to survival and reproduction, development of secondary sexual morphological characteristics, and various aspects of social behavior (Borg, 1994a; Cardwell et al., 1996; Trainor and Marler, 2001; Wingfield et al., 1990a). Importantly, at a more basic level, potent physiological impacts of steroid hormones occur via two modes: 1) steroid binding through membrane or nuclear associated receptors that up-regulate specific biochemical pathways via intracellular mechanisms, which then affect protein translation and 2) endogenous regulation of steroids via feedback loops. These levels of analyses are not possible for most fundamental investigations and proxies of steroid action must be relied upon.

Which is the most accurate representation of steroid levels that cause activational effects in an organism? The answer to this question is anything but clear-cut, because all proxies are indirect measures and have limitations. “Biomarkers” are used for assessment of endogenous steroidal metabolites present in a biological system of cells, tissues, and biofluids (Kotłowska, 2012). Often, the type of biological sample that is used as such a proxy is not based on accuracy, but rather, on feasibility (Figure 1.2). There are many different levels of endocrine analyses, ranging from direct hormone measurements to receptor densities. Additionally, steroid receptor co-activators can modulate the downstream cellular response (Charlier et al., 2006; Duncan and Carruth, 2011). When utilizing comparative approaches, there are many constraints of the organism being studied. Technological advancements have allowed for development of assays
and equipment that maximize steroid extraction, separation, recovery, detection, and sensitivity from a wide array of samples (Makin et al., 2010; Taves et al., 2011). However, the degree of invasiveness of a procedure and how often samples are collected are important considerations. The anatomical site from which a sample is collected should be reflective of the experimental question of interest, and carefully interpreted as such. This is especially critical when comparing steroid levels across a variety of sample types.

The site of action – the cell, is probably the best location to measure active steroids. Microdialysis is a technique that allows measures of steroids in the forebrain of live, behaving animals (Remage-Healey et al., 2008). In addition to sequestering steroids produced in the periphery, the brain is a remarkably heterogeneous organ that has specific sites of steroidogenic enzyme expression, steroidogenesis and sex steroid receptor expression (Arterbery et al., 2010; Carere et al., 2007; Do Rego et al., 2009; Pradhan et al., 2010b; Schmidt et al., 2008). The steroidogenic potential of the brain can be measured at specific regions or subcellular compartments by in vitro assays and is another valid proxy (Black et al., 2005; Pradhan et al., 2010a; Pradhan et al., 2010b). The evidence for synaptocrine signaling, which encompasses steroid synthesis within the presynaptic bouton and release in the synaptic cleft for rapid neuromodulation, has spurred a re-evaluation of the most widely used proxies of hormone measurements (Peterson et al., 2005; Saldanha et al., 2011). The advantage of local synthesis within a traditionally ‘target’ organ is primarily the speed of steroid action due to temporal and spatial specificity (Saldanha et al., 2011; Schmidt and Soma, 2008). Another advantage is that local synthesis reduces the need for excess hormones to be elevated system-wide due to the tremendous associated costs (Wingfield et al., 2001).
Other proxies of measurements are based on a widely known fact that centers in the hypothalamus and pituitary release regulatory hormones that stimulate the gonad or the adrenal gland to produce potent sex steroids or glucocorticoids, respectively. The traditional assumption follows that large quantities of steroids produced by endocrine organs, the ‘source’, floods the circulatory system, and hormones are then transported to specific target organs to cause downstream actions on phenotype. As a rebuttal to the accepted view that gonadal steroids also intricately control behavior, we propose that the release of hormones from the gonad is a system-wide signal that advertises or “screams” the readiness of the gonad to release gametes. We refer to this as the “Screaming Gonad Hypothesis”, and this is largely applicable to species whose mating behavior is based on reproductive cycles. Sexual selection likely acts on hormones and there are direct fitness consequences because hormones regulate gamete production. Evolutionarily speaking, the rest of the organism must be entrained to reproductive signals from the gonad, so that all efforts of the organism work towards increasing fitness. However, the gonadal function in some species is regulated by social interactions (discussed below), and rapid and fine-tuned control of behavior cannot occur unless via local modulation.

At a very crude level, the weight of gland or the tissue that synthesizes the hormone has also been measured to estimate the possible steroidogenic output. This is an indirect measure of steroid-releasing potential of that tissue. Direct measures of steroids within specific tissues have been used successfully in species of fish (Lorenzi et al., 2012) birds (Charlier et al., 2010; Schmidt et al., 2009), and rodents and provide a deep understanding of steroid function at the site of action. However, the most common route of endocrine measurement is superficial – plasma or serum is widely used because of the ease of availability from most model organisms. This measure represents the systemic levels in circulation and based on the view that steroids
produced in specific organs are released into the general circulation. Steroids are generally bound to carrier proteins, such as sex hormone binding globulins (Heinlein and Chang, 2002), and corticosteroid-binding globulin or albumin (Huddleston et al., 2007) for transport to specific target organs that express binding proteins. Hence one must also exercise caution in interpreting plasma levels because it contains concentrated steroids compared to whole blood or red blood cell, and might be an overestimation (Hiramatsu and Nisula, 1987; Taves et al., 2010).

Environmental stressors can also have differential effects on steroids and their binding to proteins (Taves et al., 2010). In addition, the part of the body that blood is collected from is also to be considered. For example, cardiac, caudal, and brachial plasma represent systemic steroid levels, while the jugular vein represents blood that is exiting the brain, and hence an indirect measure of steroids exiting the brain (Newman et al., 2008; Saldanha and Schlinger, 1997; Schlinger and Arnold, 1993). As a result factors such as half-life, rate of elimination, and rate of conversion to other active or inactive steroids might vary, based on the precise site of blood collection.

Non-invasive procedures are commonly used for diagnostic testing of disease, contamination, responses to an environmental stressor or endogenous factor, understanding the mechanism of steroid action, steroid profiling for abuse (De Jager et al., 2011; Divari et al., 2011; Heitzman, 1976); assessment of reproductive status for wild and livestock populations. Alternative measures, based on the ease of sample collection are used in such cases. Animal waste products are also routinely sampled. For example, fecal samples are used in wild or threatened populations (Borque et al., 2011). Urine samples can be easily collected from cattle (Doué et al., 2012), and amphibians (Narayan et al., 2012). Another non-invasive procedure for measuring steroids in small fish is steroids exuded in water (Sebire et al., 2007). For this
procedure, the animal is temporarily kept in a beaker containing clean water for a period of time that allows steroids to accumulate through gills, osmotic exchange through skin, and excretion. Water-borne samples are a good representation of systemic steroids because they are correlated with plasma (Gabor and Contreras, 2012). For humans, salivary hormones are often measured, and even though this is non-invasive, it has an array of limitations due to context-specific variation, that are not always controlled for (Hansen et al., 2008; Kudielka et al., 2009). Other non-invasive proxies of steroid measurement include feathers (Koren et al., 2012), hair (grizzly bears (Macbeth et al., 2010); humans (Dettenborn et al., 2012)), yolk (reptiles (Huang et al., 2013); fish (Feist et al., 1990), birds (Sockman et al., 2006), and cerebrospinal fluid (Heidbrink et al., 2010). Taken together, other than direct measures of steroids at the site of action, all other proxies must be regarded as superficial.

1.5 Endocrine manipulations

Mechanistic studies help decipher causative relationships between steroids and phenotype. Such approaches have greatly fueled our understanding of regulation of behavioral and morphological phenotypes. Steroids can be manipulated locally in particular tissues of interest or systemically. The context of anatomical location and the type of endocrine manipulation is necessary to consider based on the particular questions of interest. Classical steroidal manipulations are chronic, founded on the theory that steroids take hours or days to exert their phenotypic effects via genomic mechanisms. However, some effects of hormones occur on a much shorter time-scale, over seconds or minutes, via non-genomic mechanisms. Hence the appropriate type of manipulation and route of administration must be considered.
Again, any steroidal manipulation must be performed under well-defined environmental context relevant to the life history of the animal being studied.

1.5.1 Peripheral manipulations

Traditionally, studies on reproductive endocrinology focus separately on gonadally produced sex steroids and adrenally or inter-renally produced glucocorticoids. Early in the field of endocrinology, drastic approaches led to the finding that ‘factors’ released into circulation control phenotype. For example, in 1849, Arnold Berthold, through a series of seminal experiments on two- and three- month-old male chickens, either removed and/or transplanted testes (Quiring, 1944). Such manipulations prevented the development of secondary sexual characteristics and the expression of suites of male-typical sexual and aggressive behaviors. Terminal examination of these animals in adulthood led him to conclude that substances released by the testes into blood are transported throughout the body, and largely to the nervous system (Quiring, 1944). Accordingly, systemic manipulations to study regulatory effects of hormones have largely focused on manipulating peripheral tissues by removal of source of the hormone (gonadectomy or adrenalectomy). There are several other methods by which peripheral hormones can be manipulated. Pharmacological manipulations are often used to block the synthesis of steroids by delivery of steroidogenic enzyme inhibitors or steroid receptor antagonists can prevent the down stream effects of steroids (Nelson, 2011). Upon subsequent delivery of hormones under investigation, the rescue or restoration of normal functioning of the animal helps decipher the mechanism of steroid action. Intraperitoneal manipulations, such as injections and implants are used commonly. There are several types of implants, that can be intraperitoneal or subcutaneous (Fuenzalida, 1950), include beeswax (Pradhan et al., 2014b), pure crystalline pellets (Pradhan et al., 2014a), controlled released pellets (Fuenzalida, 1950), in
situ forming microparticle implants (Castillo-Briceno, 2013), and silastic (Damassa et al., 1977). Additionally, steroids can also be ingested via mixing in food (Remage-Healey and Bass, 2006).

1.5.2 Local manipulations

Local steroid manipulations are often effective because they occur at the site of hormonal action at the cellular level (Friedman et al., 1964). This can be accomplished by performing implants directly within the tissue in question, and has been done previously in thymus (Friedman et al., 1964) and brain (Hartmann et al., 1966; Ramirez et al., 1964). For central hormone manipulations, cannulae have been used for long-term experiments (Huddleston et al., 2006) and ICV injections have been used for short-term studies (Solomon-Lane and Grober, 2012; Tehranipour and Moghimi, 2010).

1.5.3 Limitations

Any type of experimental manipulation in vivo can have limitations. Both peripheral and local manipulation can affect an organism in various ways based on the degree of invasiveness of the procedure. Administration of hormones can produce a high degree of individual variation in the amount of systemic and local hormone levels. Treatment can effect at many different levels and differential rates of endogenous feedback loops or breakdown might be involved (Damassa et al., 1977; Pradhan et al., 2014a). Some hormones are extremely potent and activating effects of hormones occur at low levels; however, hormones do not exert effects in a dose-dependent manner (Nelson, 2011). Often, much higher doses of hormones are required for restoration than the maintenance of behavior (Damassa et al., 1977), and this might have costly physiological side-effects (Wingfield et al., 2001) and frequent delivery, such as injections, might be too invasive. Some drugs may not penetrate the blood brain barrier (Leshchenko et al., 2006), and thus central effects on behavior may not be produced (Pradhan et al., 2014b). We can also use
different manipulations to consider endocrine versus paracrine manipulations and discriminate between peripheral versus local effects. Steroids might act rapidly and hence, the environmental contextual cues may not be congruent with the endocrine context (Pradhan et al., 2014a). Different steroids also have differential rates of release, binding, breakdown, conversion, sequestration, and lipophilicity (Babuska and Pyaka, 2006). Another consequence of hormone delivery is that the intended manipulation of one hormone, such as via an enzyme inhibitor, can affect more than one hormone. Enzymes often act in more than one direction, and the same enzyme can participate in the conversion of multiple steroids. For example, administration of carbenoxolone, an 11β-hydroxysteroid dehydrogenase (11β-HSD) inhibitor, increases cortisol while decreasing 11-ketotestosterone (KT) (Pradhan et al., 2014b). Thus downstream effects could be due to the combined effects of both these hormones. This limitation is not specific to peripheral manipulations, but the resultant mechanisms under investigation must be evaluated under the appropriate endocrine context. Additionally, measures of systemic levels of hormones following peripheral manipulations are not indicative of the degree to which exogenous steroids remain elevated in specific tissues for subsequent biological effects. In L. dalli, systemic KT manipulation elevates KT differentially across different tissues (Pradhan et al., 2014a). It is likely that the elevated peripheral hormones are transported to tissue via the blood supply, but the vascular supply to the brain may not have a significant impact on total brain steroid levels (Taves et al., 2010). Some of the impacts of peripheral delivery can be circumvented via local manipulations.

1.5.4 Endocrine context in Lythrypnus dalli

Here, water-borne steroids were quantified, which are a good representation of body-wide steroids (Lorenzi et al., 2008; Rodgers et al., 2006; Wong et al., 2008). This is a non-
invasive method of collecting steroids that are exuded by fish into the surrounding water via gills and urination (Sebire et al., 2007). This method is particularly appropriate and useful for small fish due to the difficulty and/or impossibility of collecting sufficient plasma. I also quantified total extractable steroids from the brain, reproductive tissue (testes and AGS), and muscle, to evaluate local tissue steroid levels (Lorenzi et al., 2012; Schmidt et al., 2009b). For systemic steroid manipulations, pressed steroid pellets or beeswax were used. Through ICV injections, brain steroid levels were manipulated.

1.6 References
Apfelbeck, B., Mortega, K.G., Kiefer, S., Kipper, S., Goymann, W., 2013. Life-history and hormonal control of aggression in black redstarts: Blocking testosterone does not decrease territorial aggression, but changes the emphasis of vocal behaviours during simulated territorial intrusions. Front. Zool. 10, 8.


Figure 1.1: Social context in *Lythrypnus dalli*. (A) Individuals in a stable social group, form a linear social hierarchy, in which the male is the most dominant member and females are in a social environment that is inhibitory to sex change. (B) Upon male removal, individuals are in a transitioning social group, such that the alpha female is in a social environment that is permissive to sex change and the more subordinate females are in a social environment that is inhibitory to sex change. The Dominance Phase is a transitional period where status resolution takes place, after which the social hierarchies return to stability.
Figure 1.2: Proxies of steroid function. There are many different approaches to determine steroid levels in organisms.
2 CONTEXTUAL MODULATION OF ANDROGEN EFFECTS ON AGONISTIC INTERACTIONS

Devaleena S. Pradhan, Kimberly R. Connor, Elizabeth M. Pritchett, and Matthew S. Grober

Previously published in Hormones and Behavior (2014); 65(1), 47-56

2.1 Abstract

Seasonal changes in steroid hormones are known to have a major impact on social behavior, but often are quite sensitive to environmental context. In the bi-directionally sex changing fish, *Lythrypnus dalli*, stable haremic groups exhibit baseline levels of interaction. Status instability follows immediately after male removal, causing transiently elevated agonistic interactions and increase in brain and systemic levels of a potent fish androgen, 11-ketotestosterone (KT). Coupling KT implants with a socially inhibitory environment for protogynous sex change induces rapid transition to male morphology, but no significant change in social behavior and status, which could result from systemically administered steroids not effectively penetrating into brain or other tissues. Here, we first determined the degree to which exogenously administered steroids affect the steroid load within tissues. Second, we examined whether coupling a social environment permissive to sex change would influence KT effects on agonistic behavior. We implanted cholesterol (Chol, control) or KT in the dominant individual (alpha) undergoing sex change (on d0) and determined the effects on behavior and the degree to which administered steroids altered the steroid load within tissues. During the period of social instability, there were rapid (within 2 h), but transient effects of KT on agonistic behavior in alphas, and secondary effects on betas. On d3 and d5, all KT, but no Chol treated females had
male typical genital papillae. Despite elevated brain and systemic KT 5 d after implant, overall rates of aggressive behavior remained unaffected. These data highlight the importance of social context in mediating complex hormone-behavior relationships.

2.2 Introduction

Interactions between conspecifics are common in social species and are adaptive in particular environmental contexts (Wallen and Schneider, 1999). Endogenous steroids regulate these social interactions in a context dependent manner, and in a variety of vertebrates, including primates (female receptivity (Wallen and Winston, 1984)), rodents (lordosis; (Kow et al., 1978)), amphibians (female receptivity; (Lynch and Wilczynski, 2008), and fish (social competence; (Oliveira, 2009). Traditionally, hormone manipulations have been used to understand the role of steroids in the proximate regulation of behavior. However, to control for environmental variation, most of these studies use a very simplified and/or artificial social environment. As a result, the presence or absence of hormone effects on behavior may be a by-product of the limited environment or unnatural social context. In particular, the probability of increasing or decreasing the expression of behavior (e.g. aggressive, sexual) with androgen manipulation is complicated because these behaviors are often exhibited in tightly regulated windows, and dependent on the integration of several ecological, social and endogenous factors (Addis et al., 2011; Gleason et al., 2009; Hau and Beebe, 2011; Hirschenhauser et al., 2008; Pradhan and Soma, 2012).

The bluebanded goby, *Lythrypnus dalli*, is a highly social, bi-directionally hermaphroditic fish species that offers a unique opportunity to decipher the proximate mechanisms by which androgens initiate and maintain anatomical and behavioral phenotype.
These fish typically live in social groups comprised of a dominant male and a harem of females, forming a linear dominance hierarchy (Reavis and Grober, 1999). The male aggressively maintains his territory, controls access to the nest (PVC tube in the lab), and exclusively exhibits courtship jerks and parenting behavior during the breeding season (Rodgers et al., 2006). When placed in new groups, these fish usually establish a robust hierarchy within 5 d and removal of the male most often results in complete sex change of the most dominant female. The process of sexual transformation involves rapid and long-term behavioral sex change, multiple phases of reorganization of group dynamics, and changes across the entire body axis of the sex-changing individual, including gonad and external genital papilla (GP) morphology. The length to width ratio (L:W) of the GP is a reliable external indicator of sexual phenotype and exposure to KT (See Figure 2.1; explained in the Methods section). Immediately following male removal (first few minutes), the rates of agonistic interactions among the females increase while they re-establish social status relationships (Reavis and Grober, 1999). The highest-ranking female is usually the most aggressive, is not subordinated by any other female (Rodgers et al., 2007), and rapidly assumes the dominant and thus male position. Depending upon the specific group, there is substantial variation in the time over which a stable social hierarchy is re-established (“Dominance Phase”), after which there is a decline in rates of agonistic interactions (Reavis and Grober, 1999).

In addition to the reorganization of gonadal and GP morphology, complete sex change also involves changes in brain chemistry (Black et al., 2004a; Black et al., 2004b; Lorenzi et al., 2012; Reavis and Grober, 1999; Rodgers et al., 2005), which may be associated with the early changes in social behavior. Social interactions can rapidly modulate androgen levels in a variety of fish species (Oliveira et al., 2001; Remage-Healey and Bass, 2005), and thus rates of agonistic
interactions associated with sex change are likely to affect androgen levels, which in turn could modulate rates of aggression. Due to the small size of the fish, we routinely measure water-borne steroids (accumulated via excretion and leakage), and this is correlated with plasma steroids in other fish species (Gabor and Contreras, 2012; Sebire et al., 2007). Hence this is a good method to non-invasively measure systemic hormone levels. In *L. dalli*, males and females have similar levels of systemic, brain, and gonad 11-ketotestosterone (KT) (Lorenzi et al., 2012; Lorenzi et al., 2008), a potent androgen in many fish species. However, there are dramatic shifts in androgen levels in transitioning individuals throughout the sex change process. Thus, in response to the same social signal, testosterone (T) and KT levels respond in a tissue-specific manner in individuals of different status (Lorenzi et al., 2012). For example, during female to male sex change, T levels decrease and KT levels increase within the brain and gonad of the sex changing female within 24 h after male removal (Lorenzi et al., 2012), while muscle levels of both KT and T, decrease in these same animals (Lorenzi et al., 2012). In beta females (not undergoing sex change), levels of KT do not change in brain or gonads, but decrease in muscle (Lorenzi et al., 2012). Additionally, following male removal, all alpha females experience an increase in water-borne KT levels (Earley and Grober, in preparation), consistent with a “challenge” situation (Wingfield et al., 1990) resulting from the social instability (Lorenzi et al., 2012; Wingfield et al., 1990).

Androgen implants can be effectively used to induce morphological and physiological masculinization in *L. dalli*, such that only 5 d after KT implantation females have masculinized external genitalia and gonadal function (Carlisle et al., 2000). Thus, this easily measurable external phenotype can be used to effectively track other biological effects of exogenous KT manipulations and in the following experiment, we have used the known increase in GP L:W as
an external phenotypic marker of KT exposure. Steroids are most often manipulated systemically, and subsequent effects on behavior and/or morphology assume that hormones enter circulation and are then predictably delivered to “target” tissues where downstream cellular effects ultimately result in changes in morphology/behavior. The degree to which systemically elevated hormones penetrate/activate tissues, is not well documented. Possible biological effects depend on factors such as plasma carrier proteins and proximity of target and source. Different local steroid loads within tissues can result from differential synthesis, binding proteins, receptors, and rate of clearance or degradation (Adkins-Regan, 2005; Oliveira, 2009). In a previous study (Rodgers, 2007), the subordinate (beta) female, in pairs of size-mismatched females was treated with KT implants. These individuals transformed into males morphologically, but, in the face of a substantial social asymmetry, retained their female typical behavior and subordinate social status (Rodgers, 2007). Thus, in a social environment inhibitory to sex change, exogenous androgen manipulations can masculinize morphology, but do not supersede social context when it comes to the expression of social behavior.

We extend this previous study to test two additional hypotheses regarding the role of KT in regulating agonistic behavior. First, we examined whether a traditional in vivo implant treatment affected long-term systemic and tissue-specific KT loads. This question is fundamental because steroids might differentially regulate the response of target cells based on their local levels. For example, neural steroids are important for the regulation of behavior (Pradhan et al., 2010; Remage-Healey et al., 2008) and prolonged local elevation of brain KT might be essential for long-term effects on behavior. Second, we examined whether a social environment permissive to social transition, would allow for the detection of rapid and/or long-term effects of KT on agonistic behavior that were previously not observed under inhibitory
social conditions (Rodgers, 2007).

In this experiment, involving pairs of size-mismatched females, we exogenously implanted the dominant individual (alpha) undergoing sex change with KT or cholesterol (Chol, control) and then recorded rates of agonistic behavior in these pairs. To evaluate the efficacy of the implant to elevate KT and induce local biological effects, we tracked morphological changes in the GP, systemic and tissue levels of KT. With respect to effects of KT on behavior, we were primarily interested in rapid effects during the “Dominance Phase”, which is the “challenge” following male removal period (Wingfield et al., 1990) when hierarchy stability is re-established. During this time, exogenous elevation of KT may allow us to see an androgen affect because of the unique social context. Secondarily, we observed long-term rates of agonistic interactions of both the alpha and beta individuals, allowing us to gain a deeper understanding of how the effects of treatment may percolate through the social group.

2.3 Methods

2.3.1 Subjects

Wild *L. dalli* were collected (N= 63) off the coast of Santa Catalina Island, California in August 2010 (permit no. SC-10676), shipped to Atlanta and maintained in a fish facility at Georgia State University under a 14L:10D cycle. The experiment was conducted over a period of 30 d, from February to March 2011. The animals were fed twice daily, at 0800 and 1600 h with brine shrimp. After anesthetization with tricaine methanosulfate (MS-222; 0.5 mg / 100 mL H$_2$O), fish were processed under a microscope: standard length (SL) was measured, banding pattern was noted (to identify individual fish), and a photograph of the GP was obtained (Figure 2.1A; Motic Images camera, connected to a MacBook). Males and females have sexually
dimorphic GP, such that males possess a cone-shape GP with a L:W greater than 1.5 (Figure 2.1B), while females GP has a more rounded shape, and L:W around 1.0 (St. Mary 1993). A total of 21 social groups were constructed, each consisting of one male and two females (alpha and beta) and randomly assigned to either Chol or KT treatment. The males were $8.13 \pm 0.07$ mm greater in standard length (SL) compared to alphas, while alphas were $4.71 \pm 0.09$ mm greater than betas. These differences in size usually assure a linear social hierarchy in the group (Reavis and Grober, 1999). However, through behavioral assessments (see below), we determined that in two groups, the smaller female became dominant. Each group was maintained in a 40 L aquarium provided with continual biological and charcoal filtration and a PVC nesting tube. Charcoal filtration is a well-established method of removing large organic molecules, including steroids (Carlisle and Grober, unpublished results). All procedures were in compliance with the Georgia State University IACUC regulations (permit no. A09018).

2.3.2 General procedures

Social groups of one male with two females were constructed such that after male removal the dominant female would be in an environment permissive for sex change. In the presence of the alpha, the beta remained in an environment inhibitory to sex change. For each social group, males and females were allowed to form stable dominance hierarchies for 6 d prior to alpha implants and male removal. The presence of a male facilitates social hierarchy establishment, while ensuring that the alpha female does not initiate sex change. The day before the surgery (d-1), baseline behavioral observations were conducted to verify the status of each individual. On the day of the surgery (d0), following the morning behavioral observations, water-borne KT was collected from each female (1200 – 1300 h) to determine pre-implant systemic KT levels (Kidd et al., 2010; Lorenzi et al., 2008). For this procedure, the fish was
placed in a cup containing 100 mL of clean water. Following water collection, the alpha female from each group was surgically implanted with either Chol or KT. The alpha and beta females, but not the males, were then returned to the home tank. Male removal under these conditions routinely results in the alpha acquiring dominant status and rapidly initiating sex change (Reavis and Grober, 1999). Behavioral observations were conducted 2 h following the surgery and up to 4 d after implant. On d5, post-implant water-borne KT was collected, and all subjects (alphas and betas) were euthanized with an excess of MS-222. Their SL was measured, and brains were rapidly removed and flash frozen on dry ice. Prior to extraction, a photograph of the gonad was obtained in situ. Finally, muscle tissue was collected ~8 mm away from the implant location. Time from sacrifice to tissue removal did not differ between the two treatment groups (Chol = 733.3 ± 46.2 s; KT = 764.2 ± 32.45, t_{19} = 0.55, p > 0.05, d=0.25). All tissues were stored at -80°C for 4 weeks prior to hormone analysis. Due to logistical reasons the water samples were stored at 4°C for a maximum of 2 weeks until extraction. There was no time-dependent steroid degradation over this period (data not shown). Similar to a previous study (Carlisle et al., 2000), a photograph of the GP was obtained on d0, d3, and d5.

2.3.3 Steroid manipulations

Cholesterol, purchased from Sigma Aldrich (St. Louis) was used as a control because all steroids are derivatives of Chol and it has been used successfully in previous studies (Carlisle et al., 2000). Crystalline KT (purchased from Steraloids, Newport) or Chol was compressed to form pressed circular pills (Parr Pellet Press) weighing ~8 mg and 3 mm in diameter. The pills were then cut into wedge shapes under a microscope and each pellet was weighed using a Metler Toledo electronic balance (Mean weights, Chol = 0.57 ± 0.03 mg, KT = 0.58 ± 0.03 mg). Care was taken to match implant weight with the SL of the fish (Regression analysis, r^2 = 0.56; p <
0.0001, Figure 2.2), and fish were anesthetized and implanted as per (Carlisle et al., 2000). Note that while alpha females were implanted, the beta females were kept outside the tank in separate cups. For the surgery, briefly, a small ventral incision was made anterolateral to the vent, and the pellet was inserted and sealed with a cyanoacrylate adhesive, and a topical stress coat was applied over the incision site. The fish were kept moist with water during the surgery. On average, it took $483.40 \pm 43.90$ s to complete the surgery for each Chol implant and $388.30 \pm 28.60$ s for each KT implant ($t=1.85, p=0.081, d=0.81$). Postoperatively, each fish was resuscitated in a cup of fresh seawater. All fish resumed opercular beats within one minute and were monitored in the recovery cup for 15 min prior to returning to the home tank, along with the betas. On d5, at the end of the experiment, each steroid pellet was retrieved via a ventral midline incision. Each steroid pellet was in the original location of insertion and retained its original shape, but was coated with bodily fluid. Each pellet was stored in a separate vial and was weighed when dry.

2.3.4 Behavioral observations

Morning behavioral observations began at 1000 h and afternoon observations began at 1400 h, except on the day of surgery, when observations were conducted 2 h after the implant. During each 10 min session, the following agonistic behaviors were recorded: approaches (when one fish came within two body lengths of the larger individual), displacements (caused by an approach, and resulted in the approached fish swimming away rapidly). Additionally, we recorded the total time each individual spent in the nest. Two Chol groups and one KT group were excluded from all the behavior analyses due to zero rates of interactions among individuals (after d0 pm), and thus status was unable to be determined based upon our criteria. In these groups, the implanted alpha individual had taken over the nest before the behavior observations
began post surgery (d0 pm), while the betas exhibited only solicitation behaviors. Rates of behavior were determined by dividing the number of occurrences of each behavior by 10 min, which was the length of observation time (Lorenzi et al., 2012).

To gain more insight into the role of approach quality, we analyzed a composite score of aggression, “agonistic efficiency” (AgEf, Solomon-Lane, Willis, Pradhan and Grober, in review), which measures the success rate of an individual’s approaches by dividing the rate of displacements by the rate of approaches. In two cases, AgEf was incalculable because no approaches were observed. In one case, an alpha from a KT group occupied the nest for the entire observation period, while the second case involved a beta from a Chol group that occupied the nest.

2.3.5 Hormone assays

As in previous studies, hormones were extracted from the water samples using C18 solid-phase extraction columns (Sepak 3 mm, Waters) fitted to a 24-port manifold attached to a vacuum pump (Earley et al., 2006; Lorenzi et al., 2008; Rodgers et al., 2006). Hormones were extracted from tissues using C18 columns (5 mm, Waters sepak), as in previous studies (Lorenzi et al., 2012; Newman et al., 2008)). Dried water and tissue extracts were then re-suspended to yield a final volume of 350 µl (5% ethanol and 95% EIA buffer supplied by Cayman Chemical kits). To ensure that the unknown values were in the detectable range of the standard curve, each sample was further diluted with EIA buffer and final calculations were adjusted (Chol, 10x and KT, 50x). Re-suspended samples were shaken on a multi-tube vortex for 1 h before beginning the EIA procedure. All samples were assayed in duplicate, following the protocol in the package insert, with modifications and validations as noted in (Lorenzi et al., 2012). Briefly, after addition of a mouse anti-rabbit IgG monoclonal antibody and specific anti-KT enzymatic tracer
(KT-acetylcholine esterase conjugate), each plate was incubated for 18 h at 4°C on an orbital shaker. Details on the cross-reactivity of the KT assay and quality of standard curve can be found on the supplier’s (Cayman) website. Following the addition of Ellman’s Reagent, plates were read using a Victor X Multilabel Plate Reader (Perkin Elmer). As recommended by the kit insert, absorbance wavelengths between 410 and 415 nm was set and readings were taken after 60, 75, 90, and 105 min of incubation, and data from the readings with the best standard curve ($R^2 > 0.90$) was chosen for final analyses. The intra-assay variation was 5.25% and the inter-assay variation was 7.50%. All systemic steroid data are presented as ng/sample (ng/mL multiplied by 0.35 mL and adjusted for the dilution, which was the amount of EIA buffer used to re-suspend the sample), while local steroids are further corrected by weight (ng sample/mg tissue). To be conservative in our approach, we excluded two KT implanted individuals from the analyses because the KT values were 5 standard deviations away from the mean.

### 2.3.6 Statistical analyses

The data were log-transformed to achieve homogeneity of variance and normal distribution where necessary. Image J was used to measure the length and width of GP from captured images, and all data were analyzed using Prism 4.0 for Mac. To track the known morphological changes in GP length to width ratio on three days during the treatment (d0, d3, d5), a two-way repeated measures ANOVA was used to examine the effect of implant (Chol or KT). To investigate whether the implants lead to systemic changes in KT levels, we used a two-way repeated measures ANOVA for the effect of time (pre- and post-implant) in KT versus Chol implanted individuals. To investigate tissue specific KT loads (brain, gonads, and muscles) after long-term hormone treatment, a two-way repeated measures ANOVA was used to compare Chol and KT implanted fish. Significant main effects were broken down using one-way ANOVAs.
and Tukey’s Multiple Comparison tests. For the behavior analyses, the morning and afternoon observations were averaged to obtain daily variations in behavior on d-1, d1, d2, d3, and d4, because t-tests revealed no significant differences between observations on these days (p> 0.05).

As noted in the Introduction, the resolution of dominance is often very rapid in size mismatched pairs of females and so we were specifically interested in the rapid effects of the hormone treatment coupled with an environment permissive to sex change. For this reason, we conducted a priori t-tests on rates of approaches and displacements by alphas and betas on d0 pm (within 2 h of implant and male removal). To analyze long-term effects of steroid manipulation on behavior, we conducted two-way repeated measures ANOVA for implant type (KT, Chol) and at several time-points relative to the implant treatment (repeated measures of time: d-1, d0 am, d0 pm, d1, d2, d3, and d4). Specifically, we looked at time spent in the nest and aggressive behavior (approaches, displacements, and AgEf) of both, alpha and beta. Bonferroni post-hocs were conducted and the α value was adjusted by dividing 0.05 by the number of contrasts (0.007). Linear Regression analysis was used to determine the relationship between the amount of steroid absorbed (initial - final implant weight) and the level of KT in each tissue (brain, gonad and muscle). Linear Regression analysis was also used to determine the relationship between KT levels (systemic and tissue) and rates of approaches and displacements. As AgEf is a composite index of agonistic interactions, in this case we performed a t-test to compare AgEf between d0 pm and d1 for the Chol groups. For all pair-wise comparisons, Cohen’s d values were calculated using the following url: http://www.cognitiveflexibility.org/effectsize/. Linear Regression analysis was also used to examine the relationship between alpha AgEf and rates of beta approaches to alpha. As a follow-up, to examine the affects of implant type on this relationship, the beta responses (approach rate) were split binomially, based on alpha AgEf being
higher or lower than 0.5, and a Chi-square analysis was performed (this comparison allowed us to examine the role of implant type in determining the approach rate of betas to alphas that have generally high or low AgEf). Data are represented as mean ± s.e.m. and all of the tests are two-tailed.

2.4 Results

2.4.1 Effect of male removal and hormone manipulation on morphology

There was no long-term effect of implants (Chol vs. KT: F_{1,19}< 0.01, p= 0.96) or time (pre vs. post treatment: F_{1,19} = 0.75, p= 0.40) on the SL of the alphas. There were significant main effects of implant (F_{1,19}= 5.21, p= 0.034) and time (F_{2,19}= 36.40, p< 0.0001) on GP L:W ratio, as well as an interaction of type of implant treatment x time (Figure 1C, F_{2,19}= 11.65, p< 0.0001). One-way repeated measures ANOVA revealed a significant effect of time relative to surgery (Chol, F_{2,9}= 6.08, p= 0.0096; KT, F_{2,9}= 46.91, p< 0.0001). For Chol treated alphas, there was an increase in GP L:W ratio on d3 compared to d0 (q= 4.83, p< 0.01, d= 0.84), but not on other days (p> 0.05) (also see Figure 2.1A). For KT treated alphas, the greatest effect was seen on d5 (d0 vs. d3, q= 7.77, p< 0.001, d= 1.73; d0 vs. d5, q= 13.65, p< 0.001, d= 2.47; d3 vs. d5, q= 5.88, p< 0.01, d= 1.09). These results are similar to a previous study (Carlisle et al., 2000), in which morphogenesis of GP was observed on some Chol treated animals 3 d after surgery, but the effect did not persist after 5 d (see Figure 2.1A). There were no significant main effects of male removal on GP L:W ratio of betas (F_{1,19}= 0.01, p >0.05).
2.4.2 Effect of hormone manipulations on physiology

To ensure that all individuals received the implant for the entire experimental period, all the implant pellets were retrieved on d5. The final average weight of the KT pellets was lower than the Chol pellet average (Figure 2.2B; t= 2.61, p< 0.05, d= 1.40). Intraperitoneal KT implants dramatically elevated systemic KT levels (Figure 2.3A) 5 d after implant (F1,17= 21.40, p< 0.001), but pre- and post-implant KT levels were not different in Chol treated alphas (t< 0.01, p> 0.05). Systemic KT implants also elevated tissue KT levels on d5 (Figure 2.3B). There was a significant effect of implant (F1,18= 15.25, p= 0.001), tissue (F2,18= 12.94, p< 0.001), and interaction between implant and tissue (F2,18= 12.51, p< 0.001). Unpaired t-tests revealed elevated KT levels in tissues of all KT treated alphas compared to Chol (brain, t18= 3.58, p= 0.002, d= 2.72; gonad, t18= 5.51, p< 0.0001, d= 2.23; muscle, t18= 11.66, p< 0.0001, d= 7.06). There was an inverse relationship between the amount of KT absorbed and the level of gonadal KT measured (Figure 2.4, r^2= 0.49, p= 0.0168). However there was no correlation between the amount of KT absorbed and systemic, muscle, or brain KT (p >0.05).

2.4.3 Effect of hormone manipulation on behavior

First, we were interested in the effects of KT during the brief window immediately following male removal (dominance phase (Reavis and Grober, 1999)), during which female status is re-established and the most dominant individual (usually the alpha female) assumes control of the nest. Interestingly, in this experiment, there were rapid and subtle differences in aggressive behavior of both alphas and betas within 2 h of implanting the alpha (d0 pm, Figure 2.5). As predicted by status, on d0 post-implant, Chol and KT treated alphas approached betas at similar rates (Figure 2.5A, t16= 0.20, p= 0.85, d= 0.10). However, KT implanted alphas were more successful at displacing betas (Figure 2.5B, t16= 2.12, p= 0.025, d= 1.01). During the
dominance phase, betas from both treatment groups increased their approach rates from baseline (d2); but betas paired with Chol-treated alphas had increased approach rates compared to betas paired with KT-treated alphas (Figure 2.5C; t₁₆ = 2.12, p= 0.025, d= 1.01). Furthermore, betas paired with Chol-treated alphas were more successful at displacing them (Figure 2.5D, t₁₆ = 2.14, p= 0.024, d= 1.02). The day following the surgery (d1), beta displacement rate returned to baseline.

Next, we investigated the role of long-term KT treatment on agonistic interactions on the different days of the implant. A two-way repeated measures ANOVA did not reveal any long term effect of implant (approaches, F₁,₁₆= 0.79, p= 0.39; displacements, F₁,₁₆= 2.30, p= 0.15) or interaction between implant and time (approaches, F₆,₁₆= 0.09, p= 0.98; displacements, F₆,₁₆= 0.22, p= 0.92). There was an effect of time on rate of approaches (F₆,₁₆= 2.47, p= 0.053) and displacements (F₆,₁₆= 2.85, p= 0.03). However, the Bonferroni test did not allow us to identify any time point where these effects were significant (p >0.007). A two-way repeated measures ANOVA did not reveal any effect of implant on beta approaches (F₁,₁₆= 2.96, p= 0.10), but there was a significant effect on displacements (F₁,₁₆= 5.41, p= 0.03) and time (approaches, F₆,₁₆= 6.61, p< 0.001; displacements, F₆,₁₆= 4.14, p= 0.01). There was no interaction between implant and the rate of betas approaching alphas (F₆,₁₆= 2.40, p= 0.059). However, for rates of betas displacing alphas, there was a significant effect of time (F₆,₁₆= 4.02 p= 0.0012 ), type of implant given to alphas (F₁,₁₆= 5.58, p= 0.03), and an interaction between implant and time (F₁,₁₆= 4.273, p= 0.0007). More specifically, there was a difference between betas from groups of Chol and KT implanted alphas on d0 pm (t₁₆ = 4.94, p < 0.001, d= 0.74). Overall, there were no significant differences in baseline rates of pre-implant behavior between the Chol and KT treated groups (p >0.05). There were also no significant relationships between KT levels and rates of agonistic
behavior in alpha females (Figure 2.6). Linear Regression analyses did not reveal any significant relationship between pre-implant systemic KT levels and aggression on d0 (approaches, r² = 0.03, p = 0.54; displacements, r² = 0.14, p = 0.13), post-implant systemic KT levels on d5 and aggression on d4 (approaches, r² < 0.01, p = 0.99; displacements, r² < 0.01, p = 0.93), post-implant brain KT levels and aggressive interactions on d4 (approaches, r² = 0.08, p = 0.26; displacements, r² = 0.09, p = 0.23; Figure 2.6), post-implant gonad KT levels and aggressive interactions on d4 (approaches, r² = 0.10, p = 0.73; displacements, r² = 0.02, p = 0.57), and post-implant muscle KT levels and aggressive interactions on d4 (approaches, r² = 0.10, p = 0.73; displacements, r² = 0.02, p = 0.57).

Following male removal and hormone manipulations, there was a significant effect of the time an individual spent in the nest (F₁,₆ = 5.21, p < 0.001; Figure 2.7). It was only during the dominance phase that some betas had access to the nest, allowing entry (the nest was not actively defended by a dominant individual). Overall, alphas occupied the nest for significantly longer periods of time after the treatment (F₁,₆ = 25.65, p < 0.0001). There was no significant effect of the type of implant received (p > 0.05), and so the amount of time spent in the nest can be attributed to the effects of male removal.

2.4.4 Agonistic efficiency

To gain more insight into the behavioral interactions within the dyad, we examined the effectiveness of an approach by calculating the AgEf (displacements/approaches). While there was no significant difference in the AgEf of KT treated alphas before vs. after the manipulation (Figure 2.8, d0 pm vs. d1 am; t₀ = 0.77, p = 0.46, d = 0.35), the pattern among the Chol treated alphas shows a decline in AgEf during the dominance phase (within 2 h post treatment) followed by a dramatic increase in AgEf the next day (d0 pm vs. d1 am tγ = 3.13, p = 0.016, d = 1.32). This
latter increase in AgEf coincides with the decreased variation in behavior of all individuals and indicates that dominance hierarchies have been established and social stability has returned (Reavis and Grober, 1999). Although beta AgEf was not significantly different between treatment groups on d0 pm (p> 0.05; Figure 2.8B and Figure 2.9), overall rates of beta approaches from groups where alphas were implanted with Chol were dramatically elevated (Figure 2.5). To gain deeper insights into this behavioral pattern, we split individuals into two groups based on alpha AgEf scores (AgEf <0.5 vs >0.5) and plotted this against the approach rate of the beta. This analysis helps to identify whether alphas that were less effective agonistically, had betas with higher approach rates. Interestingly, groups with alphas that had lower AgEf scores all received Chol implants and were paired with betas that exhibited high rates of approaches, while groups with high AgEf alphas were much more likely to have received KT implants (Figure 2.9, \( \chi^2 \) analysis, p< 0.05). However, alphas with high AgEf received far fewer approaches from their associated betas, regardless of implant type (Figure 2.9, inset). Across all groups, there was a strong negative relationship between alpha AgEf and the rate of betas approaching alphas (\( r^2 = 0.51, p = 0.0027 \); Figure 2.9 inset). In two cases, Chol alphas had AgEf= 1, and in these cases, betas exhibited a low approach rate.

### 2.5 Discussion

These results demonstrate that implanting *L. dalli* females with KT can dramatically elevate levels of systemic and local KT 5 d after treatment (Figure 2.3). This represents one of few studies that demonstrate tissue-specific steroid increases as a result of steroid implantation (Kinci et al., 1987; Myer et al., 1979). Additionally, exogenous KT masculinized female GP morphology after 5 d, similar to the results of a previous study (Figure 2.1) (Carlisle et al., 2000).
There were effects of KT on agonistic interactions only during a brief period following male removal, during which females normally resolve status. Not only were there behavioral differences in the hormone-treated animals, but also in the reciprocal interactions with un-manipulated beta individuals in those dyads. After the animals re-established hierarchy stability, there are no long-term effects of KT on rates of aggression (d4).

2.5.1 Hormone measurements as physiological proxies

In this experiment, one major focus was to determine the efficacy of a systemic KT implant by measuring long-term increases in brain KT. Systemic steroid manipulations are a traditional approach to examine the activational effects of hormones on phenotype (behavior and morphology), but this assumes that the hormones enter circulation and are reliably delivered to “target” tissues. A systemic measure (water-borne, in this case) is informative of the overall distribution of the steroid throughout the body that can potentially permeate tissues (Lorenzi et al., 2012). We verified that water-borne (systemic) KT levels were elevated 320x in individuals with KT implants 5 d after the manipulation (Figure 2.3A). However, this measure is not indicative of the degree to which exogenous steroids remain elevated in specific tissues, and have subsequent biological effects. It is likely that the elevated peripheral KT is transported to tissue via the blood supply, but the vascular supply to the brain may not have a significant impact on total brain steroid levels (Taves et al., 2010). Previous studies demonstrate that tissues have different steroid loads resulting from synthesis and abundance of binding proteins or receptors (Adkins-Regan, 2005; Oliveira, 2009).

Significant local changes in steroid levels as a result of the implant can be a close proxy to study the activating effects of hormones. For example, the GP, a sexually dimorphic trait, is highly responsive to the long-term (5 d) KT treatment and thus represents a good bioassay to
track the effect of KT (Figure 2.1). Unfortunately, due to size limitations we were unable to measure steroids in the GP in this experiment. Interestingly, adult males (not actively parenting) and females have similar levels of systemic (Rodgers et al., 2006), brain, and gonadal (Lorenzi et al., 2012) KT. We hypothesize that differences in GP morphology are due to sexual dimorphism in androgen receptor (AR) densities and this can be a focus of future studies.

Elevations in KT levels varied across the three tissues as a result of the exogenous implant. Following KT treatment, gonads showed 92x increase, which was the highest (Figure 2.3B), while the muscle (42x) and the brain (10x) had smaller increases compared to controls. This indicates that there is differential penetration, sequestration or breakdown of KT in these tissues. We can speculate that the transitioning gonads (due to female to male sex change) have the highest numbers of AR to allow binding of KT compared to the other tissues providing a basis for the higher KT levels found in gonads in the present study. In addition, due to the closer proximity of the implant to the gonads, relative to the brain and muscle samples, there could have been a greater rate of KT entry. Interestingly, however, there was a negative relationship between the amount of KT absorbed from the pellet and the level of KT measured from the gonads of KT implanted fish (Figure 2.4, p= 0.017), but not Chol fish (p >0.05). In fact, the fish with highest amount of KT pellet absorbed had levels of gonad KT similar to Chol fish. In addition, there was no association between amount of KT absorbed and systemic, muscle or brain KT (data not shown). This could be a result feedback system operating in the gonad, but not in other tissues. These results suggest that proximity to the implant does not explain the high amounts of gonadal KT. Finally, these data demonstrate that KT implants effectively elevated brain KT, which would be essential for the display of behavioral effects.
An interesting discovery was made through our method of using pure crystalline steroids, with KT implants weighing considerably less after 5 d compared to Chol (Figure 2.2B), indicating that more KT was absorbed relative to Chol. This could be due to the differences in size, structure, polarity, and most importantly, lipophilicity of these molecules, making them differentially permeable across biological membranes (Waterhouse, 2003). Steroid conjugated antibiotics and DNA (particularly Chol) are widely used for efficient delivery of drugs to tissues, including the brain (Kichler et al., 2005; Kourtópoulos et al., 1983). It is generally accepted that Chol has a lower rate of absorption compared to androgens and estrogens in rats (Emmens, 1941; Forbes, 1941; Fuenzalida, 1950). These results identify a major limitation of using implants to manipulate steroid levels, in that there is high among tissue variation in steroid bioavailability and endogenous negative feedback loops in an organism to maintain homeostasis. In this case, such tissue specific effects could be a combination of exogenous elevations and endogenous reorganization of reproductive tissue during natural sex change.

2.5.2 Rapid and context-specific behavioral effects

We were also interested in the behavioral effects of short-term and long-term androgen manipulation. In a similar study using pairs of females (Rodgers, 2007), subordinate individuals in a dyad were given KT implants, thus treated fish were in an inhibitory social environment. In that environment, the subordinate female given the exogenous KT did not exhibit behavioral differences and did not rise to a higher status, but underwent morphological changes to the GP and gonad. Possible rapid effects in behavior were not measured in that study (Rodgers, 2007). In this experiment, we implanted the alphas with Chol or KT and placed them in a social environment permissive to sex change. There were rapid (within 2 h) and ephemeral effects of KT treatment on aggressive behavior. Alphas treated with KT and the associated betas
demonstrated behaviors as predicted by their status (Figure 2.5). Even though Chol treated alphas displayed a similar number of approaches compared to the KT alphas, fewer of these approaches led to displacements (Figure 2.5). Our results show that KT alphas were more successful at displacing the betas (Figure 2.5B). Due to the absence of the male and the resulting “challenge” situation, all the female fish generally exhibit high systemic (Earley and Grober, unpublished results) and brain KT (Lorenzi et al., 2012). It is possible that some alphas had further elevated KT above a threshold, which caused a cumulative effect of endogenous and exogenous KT and permitted the expression of successful agonistic behavior. In addition, it is also possible that the KT implanted fish responded differently to the surgery compared to the Chol animals. For example, another androgen, testosterone, has neuroprotective effects on recovery from surgery and injury (Jones et al., 2001), and KT might also have similar beneficial effects, allowing these fish to interact faster in this social context. In the future, it will be useful to monitor the recovery of these fish from anesthesia following surgery based on the measurement of other physiological responses, such as rate of opercular beats and the time taken to regain equilibrium (Solomon-Lane and Grober, 2012).

The specific behavioral patterns exhibited with androgen manipulations were only prominent during the “dominance phase” after male removal. Androgen levels do not predict aggression in established groups or status in established groups relative to unstable social hierarchies in a variety of species (Beehner et al., 2006; Oliveira et al., 2002; Ramenofsky, 1984). This finding highlights the idea that animals are sensitive to contextual information in their environment, and when coupled with the intrinsic hormonal milieu, exhibit behavioral plasticity. For example, seasonally reproducing or migratory birds exhibit temporal separation of reproductive, aggressive (associated with territory establishment), and parenting behaviors that
correlate with specific circulating or tissue levels of androgens (Ketterson and Nolan, 1992; Wingfield et al., 1990). In contrast, Tropical songbirds may not exhibit stark differences in aggression and androgens with territory establishment, but rather with maintenance of territories (Hau and Beebe, 2011). The microhabitat of a species is also critical in sparrows in the temperate regions of the Southern Hemisphere, in which highland and lowland dwellers respond differently to androgen inhibitors (Addis et al., 2011). Environmental context is also important for rodents who are more likely to win social challenges in their home cage and show resultant changes in physiology and brain sensitivity (Fuxjager et al., 2010; Fuxjager et al., 2009). Finally, social context during short-term aggressive challenges are critical in regulating urinary excretion of KT in African cichlids (Hirschenhauser et al., 2008). Thus, without the relevant environmental context, traditional endocrine manipulations often fail to have an effect on behavior.

Equally important is the fact that KT had a behavioral effect within only 2 h of treatment. In addition to the KT increase following KT implants, the alphas are likely experiencing natural physiological increases in KT as a result of being in an environment permissive to sex change following male removal (Lorenzi et al., 2012; Earley and Grober, unpublished results). However, it is necessary to be cautious with this possible interpretation because we have not measured brain KT levels within two h of the IP implant. Steroid hormones rapidly regulate social behavior in several species of vertebrates (Remage-Healey and Bass, 2006b). Within only 10 min, KT increases male advertisement calling in Gulf toadfish (*Opsanus beta*) (Remage-Healey and Bass, 2006a). Male goldfish (*Carassius auratus*) treated with testosterone increase approach rate towards females, but not males, indicating an increase in sexual behavior (Lord et al., 2009). Importantly, increased testosterone also has fitness consequences in goldfish because
it rapidly increases ejaculate volume and sperm density (Mangiamele and Thompson, 2012). In California mice (*Peromyscus californicus*), rapid effects of 17β-estradiol on male aggressive behavior only occurs under short days, but not long days, also highlighting the importance of context (Trainor et al., 2008). These rapid, non-genomic effects of steroids can occur through the activation of second messenger cascades via several cellular mechanisms (Heinlein and Chang, 2002).

2.5.3 *Alpha behavior may cause changes in beta behavior*

Treatment of alphas with KT might affect the quality of their approaches, with subsequent affects on the behavior of betas. In *L. dalli*, immediately after the disruption of hierarchy (due to male removal), females generally increase rates of agonistic interactions. However, after the status is established, the subordinate individuals reduce their rates of approaches towards males. On days when there are few eggs in the nest, the rates at which females approach males are positively correlated with rates of female solicitation behavior, most likely for spawning opportunities (Pradhan and Grober, unpublished data). However, within the social context under which this experiment was conducted, the initial approaches by the betas were not solicitations, and were likely to be for status resolution. We can explain the behavioral pattern seen here by tracking the status establishment of the alpha (the new male), following which approach rate of betas decreases. Thus AgEf can be used as a measure to determine the success rate of an alpha approach in displacing the beta. We found that groups with alphas that had low AgEf scores all received Chol implants and had betas that exhibited high rates of approaches, while groups with high AgEf alphas were much more likely to have received KT implants (Figure 2.9, \( \chi^2 \) analysis, \( p < 0.05 \), suggesting that KT affects the quality of approaches. Additionally, alphas with high AgEf received fewer approaches from their associated betas,
regardless of implant type ($r^2 = 0.51$, $p= 0.0027$; Figure 2.9 inset), demonstrating beta responsiveness to the individual behavior of their alpha, rather than the level of KT in the alpha. In two cases, Chol alphas had AgEf= 1, and the associated betas exhibited a low approach rate, again indicating that the betas are very responsive to high AgEf alphas, regardless of their steroid levels. Taken together, these data show that *L. dalli* behavior is very sensitive to social context, and composite assessments such as AgEf can be quite useful when studying social behavior (Schurch et al., 2010).

Our measures of behavior demonstrated by the betas are helpful for future experiments on social behavior and provides insights into the composite social behavior of the pair. Overall, the response of the betas raises three alternative hypotheses regarding their social interactions. First, the betas may sense the higher KT levels in alphas, or another chemical that resulted from KT effects, by an unknown chemical means, and as a result reduce their rate of approach. Second, KT exuded by the alphas into the surrounding water may also be taken up by betas. For example, in goldfish (*Carassius auratus*), androstenedione, another androgen, can rapidly transmit chemical signals with rapid behavioral effects among conspecifics (Sorensen et al., 2005), and this could also be a potential mechanism used by *L. dalli*. Third, betas may be responding to a change in the quality of alpha behavior. Fourth, interactions with the alpha may also cause changes in physiology of betas, which lead to modulation in behavioral output. As noted above, our data are most consistent with the third hypothesis, since the behavior of betas was most closely associated with the behavior of alphas, rather than the type of implant received or level of KT measured. In the future, we can measure the bi-directional relationship between hormones and behavior by also measuring hormones in the betas. For example, the degree of receptiveness of a female to a male dove’s displays can rapidly stimulate her reproductive
endocrine system, which has been termed behavioral ‘self-feedback’ (Cheng, 1983). Thus reciprocal relationships with stimulus animals or group mates provide greater insight into understanding overall social interactions, rather than only evaluating responses of manipulated individuals.

2.5.4 No long-term effects on rate of aggressive behavior

Despite the dramatically higher levels of brain KT in KT treated alphas after 5 d, rates of agonistic interactions were not higher compared to Chol treated alphas. One explanation for this observation is that after the animals have resolved status, there may be a cost to increased display of agonistic behavior. After the imposition of subordination of the lower ranking individual (Rodgers et al., 2007), we can predict greater reproductive payoffs for maintaining a basal rate of interaction, rather than energetically costly aggressive interactions. It is important to note that individuals in each social group were familiar with each other throughout the experiment (rather than using stimulus animals for short periods of time), as is the case in natural social groups. As a result, the social context used in this experiment was highly subjective for each group, and we have little control over how long status resolution takes. As stated earlier, it is likely that we missed the critical window for status resolution for three groups, which could not be included in the behavioral analyses (see Methods). The high variation in AgEf and resulting beta approach rate (Figure 2.9 inset) further demonstrates the complicated and nuanced nature of these social interactions. There are several studies in birds that demonstrate that elevated testosterone does not affect or correlate with courtship or aggressive behavior (Cramer, 2012; Day et al., 2007; Deviche et al., 2012). Our view is that the endocrine regulation of social behavior is a threshold based effect and the probability of behavior increases only when both the hormone levels exceed
threshold and the environmental stimuli are present to release particular behavioral phenotypes (Goymann et al., 2004).

2.5.5 Conclusions

A variety of studies have demonstrated and carefully examined hormone-behavior relationships (reviewed in (Nelson, 2011)). In this study, we observed the effects of in vivo systemic endocrine manipulations on multiple levels of biological organization. We demonstrated rapid effects of KT on genitalia and gonad morphology. Water-borne and local endocrine measurements demonstrated dramatic increases in tissue KT levels as a result of systemic implants. While the treatment did result in elevated brain KT levels, there was no long-term effect on rates of aggressive behavior, providing no evidence for the notion that hormones drive behavior with an “on-off” switch mechanism. Our data provide evidence for multiple gating mechanisms in the regulation of social behavior, such that hormones can have important impacts, but only under specific contextual circumstances. Thus, it is critical to consider the role of context when evaluating the effects of hormones on the expression of behavior.

2.6 Acknowledgements

We thank Madelyne Willis for help with behavioral observations and assays, Tessa Solomon-Lane, Ravi Batra, David Sinkiewicz, Mary Karom, Swathi Gannavaram, and Dr. Wilczynski for technical support. We are grateful for the grants from NSERC PGS D3, Sigma Xi, and Brains & Behavior Program at Georgia State University to DSP, NSF (IOB – 0548567) to MSG, and NSF Doctoral Dissertation Improvement Grant (1210382) to MSG and DSP.
2.7 References


Emmens, C.W., 1941. Rate of absorption of androgens and estrogens in free and esterified form of subcutaneously implanted tablets. Endocrinology 28, 633-642.


Figure 2.1: Genital papillae (GP) structure in *L. dalli* (A) three representative *L. dalli* transitioning females treated with Chol or KT on d0, d3, and d5; location of the vent is shown by the arrow, and the measurements of the length (L) and width (W) are indicated (B) a representative male GP (C) effect of implants on GP length to width ratio against time relative to steroid treatment. (n=10 Chol and n=11 KT). *p<0.05.
Figure 2.2: 11-ketotestosterone implant pellets. (A) Relationship between steroid implant weight (Chol and KT) and standard length of alpha females (B) Pre-implant, initial (d0) and post implant, final (d5) Chol and KT implant.
Figure 2.3: Levels of KT in *L. dalli* before (females) and after 5 d (transitioning males) of treatment with Chol or KT in (A) Systemic (water-borne): pre-implant is d0, post-implant is d5 and (B) Local (tissue) levels in brain, gonad and muscle of transitioning *L. dalli* on d5 (n=10 Chol and n=11 KT). **p<0.01, ***p<0.001.
Figure 2.4: Relationship between the amounts of KT absorbed by KT implanted alphas (initial – final weights of KT pills) and the level of KT in the gonad. Amount of KT absorbed negatively correlated with gonadal KT levels. (n=10 Chol and n=11 KT).
**Figure 2.5:** Rates of agonistic interactions between alpha and beta *L. dalli* relative to the time before or after alphas were treated with either KT or Chol (A) Alpha approaches beta (B) Alpha displaces beta (C) Beta approaches alpha, and (D) Beta displaces alpha. * p<0.05. (n=8 Chol and n=10 KT) Dotted lines represent the transient window of social instability that follows male removal (“the dominance phase” (Reavis and Grober, 1999)).
Figure 2.6: Relationships between rates of agonistic behavior of alphas treated with Chol or KT;
(A) Behavior and systemic KT on d0 (B) d4 behavior and systemic KT on d5, and (C) d4
behavior and brain KT on d5.
Figure 2.7: Total time spent in the nest by (A) Alphas and (B) Betas relative to time after implant. (n=10 Chol and n=11 KT); ***p< 0.001.
Figure 2.8: Agonistic efficiency of alpha and beta females. (A) Alpha agonistic efficiency (AgEf, displacements/approaches) towards betas relative to time after implant, (B) Beta AgEf towards alphas relative to time after implant (n=8 Chol and n=10 KT).
**Figure 2.9:** Categorical representation of rates of beta approaches when alpha AgEf was < or > 0.5. The numbers above bars represent the number of groups for each category. The inset is a regression of alpha AgEf against rates of betas approaching alphas after alphas were treated with either KT or Chol (N=15). Note that some individuals were excluded from this analysis due to zero rates of interaction and overlap does not allow all data points to be seen clearly.
3 WATER-BORNE AND TISSUE ENDOCRINE PROFILES OF AN
ALTERNATIVE REPRODUCTIVE PHENOTYPE IN THE SEX CHANGING FISH,
LYTHRYPNUS DALLI

Devaleena S. Pradhan, Tessa K. Solomon-Lane, and Matthew S. Grober

In press for publication in Copeia (2014)

3.1 Abstract

In the bi-directionally hermaphroditic fish, Lythrypnus dalli, two distinct male
phenotypes have been described. The more conspicuous parenting males are larger, establish
breeding territories, and display courtship, mating, and parenting behaviors. The alternative
males, called mini males, have been postulated to have a parasitic reproductive strategy, although
the behavioral ecology of mini males is not well understood. The mini male morph has been
characterized based on size and anatomical differences, including sperm-filled accessory gonadal
structures (as opposed to mostly mucous in the nesting males), consistent with parasitic male
morphs in other Gobiid species. Here, we determined the endocrine profiles of mini males to
gain further insight into their phenotype. Systemic (water-borne) 17β-estradiol (E₂)
concentrations were higher than testosterone (T), and 11-Ketotestosterone (KT) concentrations
were lowest. From a review of literature comparing breeding and parasitic males of other
species L. dalli are similar, in that mini males have higher T:KT ratios than breeding males. In
mini males, brain and reproductive tissue levels of T, E₂, and KT were higher than in the muscle.
Among all the steroids, E₂ levels were high in mini males, and in all three tissues. Data from
relative hormone levels in different tissues may lead to a better understanding of the endocrine
regulation of behavioral, physiological, and morphological correlates of male sexual polymorphism.

3.2 Introduction

Teleost fishes exhibit a wide range of strategies to maximize reproductive success, and among these, male sexual polymorphism is very common. Males of several species exhibit alternate reproductive tactics (ARTs), which enable those of poorer competitive ability to procure fertilizations (Oliveira et al., 2001a; Taborsky, 1994; Taborsky, 1998). In general, two broad categories of male morphs have been described. ‘Bourgeois’ (parenting) males are the larger, competitively superior males who monopolize resources, aggressively defend territories, court females, and, in many fish species, invest in paternal care (Taborsky, 1998). ‘Parasitic’ males do not compete for mates, instead they employ different behavioral tactics which include behaving as satellites, group mobbers, streakers, sneakers, or female mimics to steal fertilizations (Taborsky, 1998).

To date, several phenotype-typical morphological and physiological differences have been described in species with male ARTs (reviewed in (Bass and Grober, 2009). For example, parasitic males have a high gonadosomatic index (gonad weight/body weight) compared to parenting males, a strong indication of sperm competition resulting from intrasexual selection (Taborsky, 1998). In addition to the testes, the reproductive organs of many male fishes also include a lobular organ called the accessory gonadal structure (AGS), seminal vesicle, or sperm duct gland. In the Gobiidae, parenting males have an AGS that is filled with pockets of sperm immersed in a matrix of mucin (sialoglycoproteins that increase ejaculate performance and longevity by extending the duration and reducing the rate of sperm release), (Mazzoldi et al., 2005), while the AGS of parasitic males consists of densely packed sperm (Scaggiante et al.,
Differences between ARTs are also reflected in brain structure, with parenting males having greater numbers of arginine vasotocin and gonadotropin-releasing hormone neurons in the brain relative to parasitic males (Bass and Grober, 2009). Additionally, in the midshipman fish, *Porichthys notatus*, there are brain region-specific differences in glucocorticoid, mineralocorticoid (Arterbery et al., 2010a), and estrogen receptors (Fergus and Bass, 2013), as well as androgenic enzyme (11β-hydroxysteroid dehydrogenase (11β-HSD) and 11β-hydroxylase; (Arterbery et al., 2010b) expression between the two male morphs. For example, the parasitic morph (Type II) has higher mRNA levels of the glucocorticoid receptor, 11β-HSD and 11β-hydroxylase compared to the parenting morph (Type I) in the vocal-hindbrain and mid-CNS regions (Arterbery et al., 2010b). Finally, there are profound differences in circulating steroid hormones (addressed in detail below) and brain aromatase activity between male morphs in several species (Brantley et al., 1993; Schlinger et al., 1999; Knapp and Neff, 2007).

In the bi-directionally sex changing bluebanded goby, *Lythrypnus dalli*, there is evidence for the existence of two male morphs based on body size and AGS morphology (Drilling and Grober, 2005). Typically, the parenting male (usually >30 mm in standard length, SL) can be readily identified: he dominates his harem of females (usually smaller in size), defends his territory and nest, and displays courtship behavior and paternal care (Rodgers et al., 2006; Pradhan et al., 2014b). Alternative parasitic male morphs in *L. dalli* are smaller, which is why they have been called ‘mini males’. Behavioral and ecological roles of this alternative morph have not yet been described. Mini males tend to overlap in size with small females, ranging in standard length (SL) between 17 – 25 mm (Drilling and Grober, 2005), but have male-like external genitalia (genital papilla, GP). Male GPs are long and pointy (length to width (L:W) ratio >1.5), while females have a wide and rounded GP (L:W ratio is close to 1) (Carlisle et al.,
Histological analysis of the mini male gonad reveal the production of high quantities of mature sperm, indicating that these males are sexually mature (Drilling and Grober, 2005). The morphology of the mini male AGS also fits the description of the AGS in other parasitic morphs within Gobiidae that have similar mating systems (Scaggiano et al., 2006).

In this study, we measured water-borne and tissue steroid hormone levels in mini males. Steroid hormones have profound effects on behavior and morphology in vertebrates. In other species with ARTs, these males have phenotype-typical steroid levels (Brantley et al., 1993; Oliveira et al., 2001a; Oliveira et al., 2001b); therefore, it is possible that L. dalli mini males also have phenotype-typical steroid levels. There are several methods of collecting steroids in organisms, which provide insight into distinct aspects of physiology. Measures of circulating steroid hormones, presumed to be of gonadal origin, are most often used to understand the endocrine regulation of behavior. Notwithstanding the potential role of circulating hormones, there is considerable evidence of differences in sex steroid levels in circulation relative to specific target tissues where hormones act (Schmidt et al., 2008). This is particularly relevant to brain levels of steroids, as behavioral change does not always correspond with changes in circulating steroid levels (Pradhan et al., 2010). Moreover, tissue-specific androgen and estrogen production are often regulated independently (Remage-Healey et al., 2008; Pradhan et al., 2010; Pradhan et al., 2014b). In L. dalli, when the dominant male is removed from the social group, all the females in the hierarchy show different responses in the different tissues. In addition, the levels of steroid hormones are also different across tissues (Lorenzi et al., 2012). This independent regulation is important because the brain regulates behavior and responds to rapid changes in social context, while other tissues (such as the gonad) have more delayed responses (Lorenzi et al., 2012). Brain aromatase, which is responsible for converting testosterone (T) to
17β-estradiol (E$_2$), has long been thought to be critical in locally regulating the expression of behavior (Adkins et al., 1980), and neural aromatase and 11β-HSD have both been implicated in several behavioral responses that are too rapid to be regulated by genomic mechanisms in the gonad (Black et al., 2005; Pradhan et al., 2014b). Given the substantial independent data indicating the potential of both systemic and tissue steroids, measuring tissue steroid levels in addition to circulating levels will provide a deeper understanding of bioavailability, production, and mechanisms by which specific behaviors are regulated (Pradhan et al., 2014a).

Mini males are found in low abundance and their number decreases in the general population over the course of the breeding season (Drilling and Grober, 2005). Hence mini males are not the focus of most studies in our laboratory. The present study was opportunistic, because we caught a number of mini males during the breeding season, and utilized these animals to address a very specific question about the endocrine correlates of ARTs in _L. dalli_.

The goal of this study is to establish the endocrine profiles of androgens (T and 11-ketotestosterone (KT)) and E$_2$ of _L. dalli_ mini males during the mid-breeding season. While endocrine profiles of females and parenting males are well established in _L. dalli_ (Lorenzi et al., 2008; Lorenzi et al., 2012), little is known about the endocrinology of mini males. In addition to being a prohormone for E$_2$, T is also a precursor for KT, the more potent androgen of the two in many fishes (Borg, 1994). For example, systemic KT implants lead to the masculinization of female-typical GP (Pradhan et al., 2014a; Carlisle et al., 2001; Carlisle et al., 2000). Behavioral effects of KT are highly context dependent, such that it influences agonistic behavior only during social instability (Pradhan et al., 2014a), but promotes parenting behavior in males without affecting agonistic interactions (Pradhan et al., 2014b). Thus the relationships among these steroids can provide insight into their physiological roles (Black et al., 2005). In the present
study, water-borne steroids were quantified, which are a good representation of body-wide steroids (Lorenzi et al., 2008; Rodgers et al., 2006; Wong et al., 2008). This is a non-invasive method of collecting steroids that are exuded by fish into the surrounding water via gills and urination (Sebire et al., 2007). This method is particularly appropriate and useful for small fish due to the difficulty and/or impossibility of collecting sufficient plasma. We also quantified total extractable steroids from the brain, reproductive tissue (testes and AGS), and muscle, to evaluate local tissue steroid levels (Schmidt et al., 2009; Lorenzi et al., 2012). Based on previous work on *L. dalli* (Lorenzi et al., 2012), we predict that levels of steroids will be different in all the tissues.

### 3.3 Methods

#### 3.3.1 Field protocol

The habitat of *L. dalli* ranges from the benthic rocky reefs of the Gulf of California, Mexico, to Morro Bay, California (Miller and Lea, 1972). Free-living *L. dalli* live in mixed sex groups. Fish were captured in association with the crowned urchin, *Centrostephanus coronatus*, off the coast of Santa Catalina Island, California between July 9 – 17, 2009 using SCUBA diving and hand nets. Fish were then transferred to Nalgene bottles for transport to the boat, where they were then transferred to a bucket filled with fresh sea water. Within 90 min of capture, fish were brought back to the laboratory at USC Wrigley Institute for Environmental Studies.

Due to the lack of dramatic sexual dimorphism in this species, it is difficult to identify mini males in the field (see (Drilling and Grober, 2005) for population estimates during the breeding season), so verification requires microscopic examination of the GP. To confirm phenotype, fish were immediately anesthetized (in water containing tricaine methanesulfonate MS-222; 0.5 mg/ 100 mL seawater) upon arriving back at the laboratory, and sex of the fish was
determined based on the morphology of the GP (Drilling and Grober, 2005). The SL was measured with a vernier caliper, and a digital photograph of the GP was obtained using an external camera that transferred images from the microscope to a laptop computer (Motic Images Software system running on a MacBook). A fish was considered a mini male (n=9) if its SL was between 17 – 25 mm and if it had a male-like GP (L:W ratio >1.5), a male-like appearance (cone-like, not rounded like females), and not transitional (Drilling and Grober, 2005; Pradhan et al., 2014a). The subject was immediately resuscitated in a cup of fresh seawater and transferred to another beaker for hormone collection. This method of collection of steroids immediately after recovery from anesthesia does not affect systemic (water-borne) cortisol levels, indicating that this procedure does not represent a significant stressor for L. dalli (Solomon-Lane and Grober, 2012).

3.3.2 Steroid collection, extraction, and enzyme immunoassays

Systemic sex steroid levels were determined by measuring water-borne steroid concentrations. Water-borne hormone samples were collected from each subject for a 2 h period between 1400 – 1700 h, according to the protocol described previously (Lorenzi et al., 2008). The only difference was that we doubled the collection time from 1 h in the previous study to accommodate the possibly slower rate of steroids being exuded into water, considering that mini males are smaller (hence have a lower surface area) than nesting males and females. Briefly, each fish was placed in a 200 mL polypropylene beaker (cleansed with 100% ethanol, distilled water, and seawater prior to use) containing 100 mL fresh seawater, obtained from the tap supplying ocean water to holding tanks. To maintain a constant temperature during the sampling period, the beaker was placed in a water table with about 2 cm of flowing seawater. Individuals being sampled were in visual contact with each other. At the end of the sampling period, the
water was poured through a net (rinsed with clean seawater) into a clean 200 mL polypropylene beaker.

Hormones were extracted from the water sample using C18 solid-phase extraction columns (Lichrolut RP-18, 500 mg, 3.0 ml; Merck) fitted to a 12-port vacuum manifold. This procedure has been validated in previous studies (Earley et al., 2006; Rodgers et al., 2006; Lorenzi et al., 2008). The extraction procedure was completed in two segments. First, water-borne steroid samples from the beakers were adsorbed to the C18 columns, both ends of each column were sealed with parafilm and they were then stored at -20°C prior to shipment to Georgia State University (GSU; Atlanta, GA). Second, the extraction process was completed at GSU according to previous studies (Lorenzi et al., 2008; Pradhan et al., 2013). The eluates were collected in 13 x 100 mm borosilicate test tubes and evaporated until dry under a gentle stream of nitrogen at 37°C.

Immediately following the 2 h sampling of systemic steroids, subjects were euthanized with an over-dose of MS-222 (1 mg / 100 mL sea water) and tissues were immediately harvested. Brain, muscle tissue (located around the caudal penduncle), and reproductive tissues (testes and AGS) were collected and frozen rapidly on dry ice. Gross anatomical examination confirmed the presence of fully developed testes. Both testes and AGS were collected, as they comprise the total reproductive tissue in males and because both these tissues are known to produce sex hormones (Lahnsteiner et al., 1993; Lorenzi et al., 2012). Dissection times for each individual ranged between 6 – 8 min. Tissue samples were then stored at -80°C for 6 weeks, shipped to GSU on dry ice, and stored again at -80°C, until further processing. Prior to extraction, each tissue sample was weighed and homogenized in 350 µL ice-cold buffer (brain and reproductive tissues in 0.1 M phosphate buffer; muscle, 0.1 M borate buffer) and 1500 µL
HPLC grade methanol (denatures proteins) was added to stop steroidogenic enzyme activity. Steroids were then extracted from tissues according to previously described methods (Pradhan et al., 2014a; Newman et al., 2008). The final eluates were collected in 13 x 100 mm borosilicate tubes and dried in a gentle stream of nitrogen at 37°C.

Dried water-borne and tissue extracted steroids were then re-suspended to yield a final volume of 350 µL (5% ethanol and 95% EIA buffer supplied by Cayman Chemical kits: T: 582701, KT: 582751, E₂: 582251), which was enough for 3 hormone assays per tissue. Re-suspended samples were shaken on a multi-tube vortex for 1 h before beginning the EIA procedure. Each EIA for T, E₂ and KT was performed in duplicate, following the manufacturer’s protocol. Validations for all these assays have been previously published (Lorenzi et al., 2008). All the tracers were provided in the Cayman Chemical kit for each steroid and was a steroid-specific acetylcholine esterase conjugate (all the details are provided in the manufacturer’s protocol). Some modifications from the assay manufacturer’s protocol as follows: first, following addition of the tracer and antiserum, incubations (T, 2 h; E₂, 1 h; KT, 18 h) were performed at 4°C on an orbital shaker; second, the plate was read at 45, 60, 75, and 90, min following the addition of Ellman’s Reagent, and the set which yielded the best standard curve ($r^2 > 0.9$) was used for further analysis of sample data. Intra-assay variations for T, E₂, and KT were 5.22%, 5.95%, and 2.68% respectively. Systemic steroid concentrations were normalized to per hour of release. Lastly, all systemic steroid data are presented as pg/sample/hr (pg/mL multiplied by 0.35 mL, which was the amount of EIA buffer used to re-suspend the sample), while tissue steroids are further corrected by weight (pg sample / mg tissue).
3.3.3 Statistical analyses

Statistical analyses were conducted using Prism 4.0 for Mac OS X. Regression analysis was used to determine the relationship between GP ratio and SL. A within subjects design was used to examine the concentrations of steroids in each individual. For systemic steroids, the data did not fit the homogeneity of variance assumptions for parametric statistical tests, so we used Friedman’s test. For tissue steroids, data were transformed [log (y)] to achieve homogeneity of variance. A two-way repeated measures ANOVA was then conducted with tissues (brain vs. reproductive tissue vs. muscle) and steroids (T, E₂, and KT) as within subject variables. Significant main effects were further analyzed by Wilcoxon-signed rank or Bonferroni tests. For each hormone, one-way repeated measures ANOVAs were used to analyze differences in tissues, and significant results were followed by Tukey tests. Regression analyses were performed to examine (1) the relationship between water-borne (systemic) and tissue (local) concentrations of steroids (2) the relationship between systemic steroid concentration and GP ratio (3) the relationship between tissue steroid concentrations and GP ratio and (4) the relationship between pairs of water-borne and tissues steroids. To allow for robust between study and between species comparisons, we looked at ratios of hormones within studies (analyzed by one-way repeated measures ANOVA) and then compared these ratios between studies (see Figure 3.3 and 3.4). For example, we computed the ratio of T:KT levels in the present study and estimated T:KT based on previously published hormone levels in nesting male and female L. dalli and in other species. Caution must be exercised when interpreting the ratio data estimates across studies because the relative averages from published work were used. All data (except for Figure 3.3 and 3.4) are represented as mean ± SEM, and α was set at 0.05.
3.4 Results

3.4.1 Water-borne steroids

There were significant differences among concentrations of systemic sex steroids (Friedman’s F = 18.00, p < 0.0001; n = 9 Figure 3.1A). Post hoc Wilcoxon signed rank tests revealed that E\(_2\) concentrations were significantly higher than T and KT (p = 0.004), and T concentrations were significantly higher than KT (p = 0.004).

3.4.2 Tissue steroids

Two-way repeated measures ANOVA revealed significant main effects of tissue (F\(_{2,24} = 21.38, p < 0.0001\); Figure 3.1B) and steroid hormone (F\(_{2,24} = 37.71, p < 0.0001\)), but there was no interaction (F\(_{2,24} = 0.93, p = 0.46\)). Post-hoc Bonferroni tests revealed no differences between T and E\(_2\) levels in any tissue (p > 0.05). Levels of both T and E\(_2\) were significantly higher than KT in all three tissues (T; brain: t = 4.24, p < 0.001; reproductive tissue: t = 4.26, p < 0.001; muscle: t = 3.54, p < 0.01; E\(_2\); brain: t = 3.69, p < 0.001; reproductive tissue: t = 5.25, p < 0.001; muscle: t = 4.89, p < 0.001; Figure 3.1B).

Levels of T in tissues were significantly different (F\(_{2,8} = 8.41, p = 0.0026\); Figure 3.1B). There were no differences in T between brain and reproductive tissue (p > 0.05), but levels of T in brain and reproductive tissue were higher than in muscle (brain vs. muscle: q = 5.80, p < 0.01; reproductive tissue vs. muscle: q = 4.02, p < 0.05). Tissue E\(_2\) levels also varied significantly (F\(_{2,8} = 11.41, p = 0.0009\); Figure 3.1B). Again, there were no differences in E\(_2\) between brain and reproductive tissue (p > 0.05), but both brain and reproductive tissue were higher than muscle (brain vs. muscle: q = 5.27, p < 0.01; reproductive tissue vs. muscle: q = 6.29, p < 0.01). Levels of KT in the tissues were also significantly different (F\(_{2,8} = 5.58, p = 0.0145\); Figure 3.1B). While KT was not significantly different between brain and reproductive tissue, and
between reproductive tissue and muscle (p > 0.05), brain KT was significantly higher than muscle (q = 4.68, p < 0.05).

### 3.4.3 Regression analyses

Of all the steroids, there was a significant relationship between brain KT and water-borne KT ($r^2=0.56$, p=0.02); however, there were no other significant relationships between any other water-borne steroids and tissue steroids (Table 3.1). There were also no significant relationships between steroids (water-borne and tissue) and SL of mini males (Table 3.2). Systemic levels of the three sex steroids were positively correlated with GP ratio (Figure 3.2). For tissues, brain KT levels positively correlated with GP ratio (Table 3.3).

### 3.4.4 Relationship between steroids

In water-borne samples and in individual tissues, T:KT was the highest and significantly different from both T:E$_2$ and KT:E$_2$ (p < 0.01) Figure 3.3 and 3.4).

### 3.5 Discussion

This was an opportunistic study that allowed us to investigate water-borne and tissue levels of T, KT, and E$_2$ in mini males of *L. dalli*. This is the first study to address the alternative male morph in this species since its original description, which was based on external morphology / gonad histology (Drilling and Grober, 2005). We did not study nesting males and females in the present study because we inadvertently caught mini and we identified them only upon examination in the laboratory. The approach here is to examine the endocrine traits (e.g., tissue and water-borne steroid hormones) in mini males to see if this species shows the suite of endocrine relationships that is well established for fish species with ARTs (Bass and Grober,
These data provide insights into the behavioral, physiological and morphological differences of these alternative morphs.

3.5.1 Water-borne steroids

Similar to females and parenting male *L. dalli* (Lorenzi et al., 2008), T was the predominant androgen in mini males, while KT was lower (Figure 3.1). As a result, water-borne T:KT ratio was highest in mini males (Figure 3.4). Of the two androgens, KT is considered more potent in teleosts (Borg, 1994), and in general, KT levels tend to be higher in parenting males, perhaps for the regulation of male-specific courtship and parenting behavior (Rodgers et al., 2013; Pradhan et al., 2014b). This observation is consistent with studies from several phylogenetically distant species, such as plainfish midshipman (Brantley et al., 1993), bluegill sunfish (Knapp and Neff, 2007), and round gobies (Marentette et al., 2009). The ratio of T to KT is generally higher in parasitic than parenting males for all of the species reported in Figure 3.4. Of the three steroids measured, E$_2$ showed both the highest mean levels and the highest variation (Figure 3.1, and 2B). In other species, circulating E$_2$ levels are seldom measured and tend to be undetectable in nesting and parasitic males (e.g. in plainfish midshipman (Brantley et al., 1993)). However, the timing is important to consider among seasonal species, and in fact, E$_2$ was detectable in parenting male plainfish midshipman in a more recent study during one sampling period (Sisneros et al., 2004). In bluegill sunfish, *Lepomis macrochirus*, many parenting males have non-detectable E$_2$ and parasitic males also have generally low E$_2$ compared to females (Knapp and Neff, 2007). For these reasons, we were unable to include E$_2$ in the ratio calculations. Finally, an interesting insight from using ratios for these analyses is that we compare across different sample collection and assay protocols (e.g. water-borne versus plasma). In mini males, T:KT was higher than both T:E$_2$ and KT:E$_2$, consistent with the high E$_2$ levels in
Interestingly, KT:E₂ was lower compared to females, which is probably because while females have high KT, mini males have lower KT and higher E₂. Taken together, high systemic T:KT in mini males, mainly attributed to low water-borne KT, in comparison with parenting males is consistent with the hypothesis that elevated KT is critical for regulating male parenting in \textit{L. dalli} (Rodgers et al., 2006; Pradhan et al., 2014b), and that there are morph-specific systemic levels of sex steroids in \textit{L. dalli}.

3.5.2 \textit{Tissue steroids}

A comparison of tissue-specific levels of T, E₂, and KT in \textit{L. dalli} mini males provides insights into the source of these hormones. If reproductive tissue is the primary producer of sex steroids, then levels should be highest in reproductive tissue. However, it is clear from the data that in mini males, both brain and reproductive tissues have similar levels of all three hormones (Fig. 1B), and these levels were, in general, higher than in muscle tissue. In contrast to the systemic data, T:KT was lower in brain, reproductive tissue, and muscle of mini males compared to nesting males and females (Figure 3.3). This trend is also consistent with gonadal T:KT in peacock blennies, \textit{Salaria pavo} (Oliveira et al., 2001a). In plainfish midshipman, the expression of mRNA coding for 11β-hydroxylase and 11β-HSD, the enzymes that convert T to KT, is higher in the central nervous system of parasitic males compared to parenting males, while these patterns are reversed in the gonad and sonic muscle tissue (Arterbery et al., 2010b). Measuring the rate of \textit{in vitro} 11β-HSD activity in these tissues might further provide insights into the relationship between T and KT, because tissues can also sequester hormones via binding to receptors (Shi et al., 2012).

Not only are there tissue-specific steroid levels, the amount of each steroid is also different in each tissue. These results are important because they question the assumption that
sex steroids are mainly produced by reproductive tissues and underscore the importance of the specific proxy of steroid measurement being chosen to evaluate hormone-behavior relationships. In *L. dalli* females, ovaries are likely to be the source of high systemic E$_2$ (Lorenzi et al., 2008; Lorenzi et al., 2012) because gonadal aromatase activity is higher than in the brain (Black et al., 2005). While the predominant source of E$_2$ is less clear in mini males, aromatase activity is high in diencephalon-midbrain and vocal hindbrain areas of parasitic (Type II) plainfish midshipman morph, suggesting a possible neural origin (Schlinger et al., 1999). In mini males, brain and reproductive tissue levels of T, E$_2$, and KT were similarly higher than in the muscle, but it is unlikely that the muscle produces steroids because it has relatively low levels of all steroids. Taken together, these results suggest that both the reproductive tissue and the brain could be significant sites of steroid production. Further studies of tissue expression and activity of steroidogenic enzymes in *L. dalli* would provide insight into the relationship between the sex hormone production and use in peripheral tissues (Pradhan et al., 2014b). Manipulation of steroids in mini males, as in previous studies involving alternative male morphs from other species (Goncalves et al., 2007; Oliveira et al., 2001b), will further elucidate the control of mini male phenotype.

Traditionally, gonads are assumed to be the source of sex steroids in circulation and target organs (Borg, 1994; Magee et al., 2006). Recent studies question whether tissues are passive recipients of sex steroids (via receptors), and/or also capable of synthesizing steroids *de novo* (Schmidt et al., 2008). Tissue-specific aromatization of circulating T has long been considered to be the mechanism for high local levels of E$_2$ in organs such as the brain (Adkins et al., 1980). In addition, recent studies provide evidence for androgen synthesis in tissues other than gonad, such as expression and activity of steroidogenic enzymes (Arterbery et al., 2010b;
Do Rego et al., 2009; London et al., 2009; Pradhan et al., 2010). Thus, it is important to consider the endocrine profiles of other tissues in the regulation of sex-specific reproductive phenotype. Specifically, steroid loads in brain tissues may provide key insights into the regulation of morphotypical reproductive behavior.

3.5.3 Water-borne versus tissue steroids

In this study, there was no relationship between water-borne and tissue sex steroids, except for brain KT and systemic KT (Tables 3.2 and 3.3). If the traditional dogma that gonads are the primary site of steroid synthesis is true, then systemic levels should closely track gonadal steroid production or levels of hormone extracted from reproductive tissue, and this relationship should hold true for other target tissues as well. Thus, it is possible that the brain produces KT, which might account for high systemic KT. Based on previous studies, both males and females from *L. dalli* social groups in the laboratory have high KT in the brain (Lorenzi et al., 2012) and brain KT can also be modulated to affect behavior (Pradhan et al., 2014b). Interestingly, the pattern of steroid levels in muscle matches the pattern of water-borne steroid levels (Figure 3.1 and Figure 3.3), suggesting that muscle may represent a passive sink for steroids synthesized in other tissues and, therefore, might serve as a useful metric for systemic steroid levels.

3.5.4 Steroids versus morphology

There was no relationship between SL and any water-borne or tissue sex steroid in mini males (Tables 3.2 and 3.3), similar to a previous study for KT in nesting male and female *L. dalli* (Rodgers et al., 2006). In a cooperatively breeding cichlid, *Neolamprologus pulcher*, there is no relationship between SL and androgens (Bender et al., 2006). This finding is contrary to bluegills, where circulating E$_2$ and SL are negatively correlated (Knapp and Neff, 2007).
Systemic steroids were all positively correlated with GP ratio; however, E₂ had the strongest relationship \( r^2 = 0.82 \); Figure 3.2). Except for brain KT, there were no relationships between tissue steroids and GP ratio (Table 3.3). Visual inspection of the data reveals that two mini males have a high GP ratio, and these two males also have higher E₂ levels (Figure 3.2). Due to the limited sample size in this study, we are unable to confirm the presence of a bimodal pattern, but we can speculate that there could be two different parasitic morphs.

3.5.5 Speculation on behavior

Even though the endocrine profiles seem to be morph-specific (Figure 3.3 and 3.4), these data do not help elucidate the strategy that mini males take in order to obtain surreptitious fertilizations. The high variation in E₂ levels, suggest a possibility of a bimodal distribution in E₂ levels and this can be confirmed in future studies with a greater sample size. Territorial males generally spawn with only one female at a time, and their nesting sites are generally in small crevices or tubes, which reduce the likelihood of sneaking or streaking strategies. Like small *L. dalli* females (18 – 24 mm SL), mini males might be less site-attached (Lorenzi et al., 2012) and, thus, could be adopting female mimicry. Under field conditions, it is challenging to reliably identify specific individuals as mini males because mini males are female-like in size and appearance. As a result, it is difficult to study the behavioral characteristics of these male morphs. However, mini male sized fish have never been observed defending a territory or dominating a harem of females. Preliminary studies in the laboratory have revealed that when placed in a social group consisting of a nesting male and several females, mini males either remain mini males or transform to females (Lorenzi and Grober, unpublished data).
3.5.6 Conclusions

Here, we establish the endocrine profiles of mini males and these physiological data are consistent with the previously published, morphologically-based description of *L. dalli* mini males. We show that brain KT is correlated with systemic KT and systemic KT is correlated with GP morphology; illustrating the importance of KT in these analyses. Out of the three steroids studied, only systemic and brain KT levels were associated. These data will help inform future studies on the role of hormones in mediating or responding to the social dynamics of mini males. Determining the local hormone profiles of tissues allows for more comprehensive understanding of endocrine regulation and corresponding phenotype. For example, data from tissue (e.g., local) can provide insight into where the site of action of steroids is and the mechanisms by which steroids have their phenotypic effects.

3.6 Acknowledgements

We thank D. Sinkiewicz and V. Smith for help in the lab and field, W. Wilczynski for technical support, and the staff and facilities at University of Southern California’s Wrigley Institute for Environmental Studies. Removal of animals from the wild was authorized by California Department of Fish & Game permit # SC-10676. All procedures were in compliance with Georgia State University IACUC regulations (permit # A09018). This work was supported with grants from Natural Sciences and Engineering Research Council of Canada Post Graduate Scholarship D3 and Sigma Xi to DSP, Brains & Behavior Program at Georgia State University to DSP and TKSL, National Science Foundation (IOB – 0548567) to MSG, and National Science Foundation Doctoral Dissertation Improvement Grant (1210382) to MSG and DSP.
3.7 References


Table 3.1: Relationship between water-borne and tissue sex steroids in *L. dalli* mini males.

Results of simple regression analysis. n=9 per group. *p<0.05
Table 3.2: No significant relationship between sex steroids and standard length in *L. dalli* mini males.

<table>
<thead>
<tr>
<th>Steroid Hormone</th>
<th>Water-borne</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Brain</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Reproductive tissue</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Muscle</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t^2$</td>
<td>$E_{(4,7)}$</td>
<td>$p$</td>
<td>$t^2$</td>
<td>$E_{(4,7)}$</td>
<td>$p$</td>
<td>$t^2$</td>
<td>$E_{(4,7)}$</td>
<td>$p$</td>
<td>$t^2$</td>
<td>$E_{(4,7)}$</td>
<td>$p$</td>
<td>$t^2$</td>
<td>$E_{(4,7)}$</td>
<td>$p$</td>
<td>$t^2$</td>
<td>$E_{(4,7)}$</td>
<td>$p$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.12</td>
<td>0.92</td>
<td>0.37</td>
<td>0.06</td>
<td>0.49</td>
<td>0.51</td>
<td>&lt;0.01</td>
<td>0.01</td>
<td>0.94</td>
<td>0.02</td>
<td>0.17</td>
<td>0.69</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17β-estradiol</td>
<td>0.03</td>
<td>0.22</td>
<td>0.66</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.99</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.97</td>
<td>0.01</td>
<td>0.10</td>
<td>0.76</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11-ketotestosterone</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.98</td>
<td>0.16</td>
<td>1.30</td>
<td>0.29</td>
<td>0.01</td>
<td>0.06</td>
<td>0.81</td>
<td>0.07</td>
<td>0.54</td>
<td>0.49</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results of simple regression analysis. $n=9$ per group.
Table 3.3: Relationship between tissue steroid levels and genital papilla length to width ratio in mini males of *L. dalli*.

<table>
<thead>
<tr>
<th>Steroid Hormone</th>
<th>Brain</th>
<th>Reproductive tissue</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r^2$</td>
<td>$F_{(1,7)}$</td>
<td>$p$</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.34</td>
<td>3.59</td>
<td>0.09</td>
</tr>
<tr>
<td>17β-estradiol</td>
<td>0.09</td>
<td>0.70</td>
<td>0.42</td>
</tr>
<tr>
<td>11-ketotestosterone</td>
<td>0.81</td>
<td>28.20</td>
<td>0.001**</td>
</tr>
</tbody>
</table>

Results of simple regression analysis. n=9 per group. **p<0.01
Figure 3.1: Water-borne (A) and tissue (B) levels of sex steroids (mean ± S.E.M) in mini males (n=9). Repro., reproductive tissue (testes + AGS). Different letters above bars indicate that the means are significantly different from each other; *p<0.05, **p<0.01.
Figure 3.2: Linear regressions between water-borne (A) testosterone, (B) 17β-estradiol and (C) 11-ketotestosterone levels and genital papilla morphology (expressed as Length:Width ratio) in *L. dalli* mini males (n=9). Averages reported in (Lorenzi et al., 2012) were used to estimate ratios for females and parenting males. T, testosterone; E₂, 17β-estradiol; KT, 11-ketotestosterone.
**Figure 3.3:** Relative tissue sex steroid ratios from (A) brain (B) reproductive tissue, and (C) muscle of *Lythrypnus dalli* parenting males, females and mini males. For mini males, mean ± SEM is reported.
**Figure 3.4:** Relative systemic sex steroid ratios from four species of Teleost fish (A) *Porichthys notatus*, plainfish midshipman, (B) *Parablennius sanguinolentus parvicornis*, rock-pool blenny, (C) *Lepomis macrochirus*, bluegill sunfish, and (D) *Lythrypnus dalli*, bluebanded goby.

Averages reported in (Lorenzi et al., 2008; Knapp and Neff, 2007; Oliveira et al., 2001; Brantley et al., 1993; Sisneros et al., 2004) were used to estimate ratios. Data for KT are not available for females of some species. T, testosterone; E$_2$, 17β-estradiol; KT, 11-Ketotestosterone, N.D., non-detectable.
4 A MECHANISM FOR RAPID NEUROSTEROIDAL REGULATION OF PARENTING BEHAVIOR

Devaleena S. Pradhan, Tessa K. Solomon-Lane, Madelyne C. Willis, and Matthew S. Grober


4.1 Abstract

While systemic steroid hormones are known to regulate reproductive behavior, the actual mechanisms of steroidal regulation remain largely unknown. Steroidogenic enzyme activity can rapidly modulate social behavior by influencing neurosteroid production. In fish, the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) synthesizes 11-ketotestosterone (KT, a potent teleost androgen) and deactivates cortisol (the primary teleost glucocorticoid), and both of these steroid hormones can regulate behavior. Here, we investigated the role of neurosteroidogenesis in regulating parenting in a haremic bidirectionally hermaphroditic fish, *Lythrypnus dalli*, where males provide all requisite parental care. Using an *in vitro* assay, we found that an 11β-HSD inhibitor, carbenoxolone (CBX), reduced brain and testicular KT synthesis by ≥90%. We modulated neurosteroid levels in parenting males via intracerebroventricular injection of CBX. Within only 20 min, CBX transiently eliminated parenting behavior, but not other social behavior, suggesting an enzymatic mechanism for rapid neurosteroidal regulation of parenting. Consistent with our proposed mechanism, elevating KT levels rescued parenting when paired with CBX, while cortisol alone did not affect parenting. Females paired with the experimental males opportunistically consumed unattended eggs, which reduced male reproductive success by 15%, but some females also exhibited parenting behavior, and these females had elevated brain
KT. Brain KT levels appear to regulate the expression of parenting behavior as a result of changes in neural 11β-HSD activity.

4.2 Introduction

Steroid hormones are critical proximate determinants of reproductive success in vertebrates because they regulate fundamental aspects of life history and phenotype (Borg, 1994; Cardwell et al., 1996; Trainor and Marler, 2001; Wingfield et al., 1990). Many groups of vertebrates exhibit temporally distinct periods of mating and parenting during the breeding season, and predictable differences in systemic levels of steroid hormones are observed during these different phases of reproduction (Wingfield et al., 1990). In some species, territory defense, mating, and parenting temporally overlap, and in these cases, a clear relationship between specific steroid hormones and behavior may not be as evident (Ketterson and Nolan, 1992; Rodgers et al., 2006). In such cases, rapid and/or local changes in steroid hormone levels might control particular reproductive behaviors. The specific mechanisms by which steroid hormones regulate parenting are largely unknown. Chronically high systemic levels of androgens and glucocorticoids have been associated with adverse effects on parenting in birds (Ketterson and Nolan, 1992; Wingfield et al., 2001) and primates (Gray et al., 2002; Muller et al., 2009; Saltzman and Abbott, 2009). In some rodents and fish, however, high systemic androgens are positively associated with parenting (Marler et al., 2003), and glucocorticoids do not appear to inhibit parenting (Magee et al., 2006; O'Connor et al., 2009). Such opposite patterns suggest that information about systemic steroid levels is incomplete to explain variation in behavior, and recently, it has become clear that rapid behavioral responses to the environment can be regulated by steroids synthesized de novo from cholesterol in the brain (Pradhan and
Soma, 2012; Remage-Healey et al., 2009; Remage-Healey et al., 2008; Trainor et al., 2003) via steroidogenic enzymes and co-substrates present in brain cells (Do Rego et al., 2009; Payne and Hales, 2004).

Steroidogenic enzymes are active in the brain and function via evolutionarily conserved mechanisms in all groups of vertebrates (Do Rego et al., 2009). In many species, neural steroidogenic enzymes are sensitive to changes in seasons (Pradhan et al., 2010), life history stages (Black et al., 2005; Soma et al., 2003), and sexual behavior (Ball and Balthazart, 2004). Neural steroidogenic enzyme expression also varies during territory acquisition, courtship, and parenting (Soma et al., 2003; Trainor et al., 2003). In fish, testosterone (T) can be converted to 17β-estradiol (E₂) via aromatase and to 11-ketotestosterone (KT, a potent androgen) via the sequential action of 11β-hydroxylase and 11β-hydroxysteroid dehydrogenase (Figure 4.1) (Borg, 1994). 11-Ketotestosterone cannot be converted to any other metabolically active steroid hormone. These steroidogenic enzymes (aromatase, 11β-H, 11β-HSD) are expressed in both gonadal and neural tissue (Arterbery et al., 2010; Jeng et al., 2012; Rasheeda et al., 2010). 11β-Hydroxysteroid dehydrogenase also catalyzes the conversion the primary potent glucocorticoid cortisol to cortisone, which has low potency. Therefore the regulation of 11β-HSD is critical because increases in its activity may result both in local elevation of KT and reduction of cortisol (Perry and Grober, 2003), and each of these changes could increase reproductive success, since these steroid hormones have been suggested to regulate parenting and aggression. This dual action of 11β-HSD might function as a switch between behavioral states, and this idea can be directly tested in fish due to the position of 11β-HSD in the steroidogenic pathways for the major glucocorticoid and androgen (Consten et al., 2001; Knapp, 2003; Perry and Grober, 2003).
The bluebanded goby, *Lythrypnus dalli*, is a serially hermaphroditic marine fish that offers an ideal opportunity to understand mechanisms regulating parenting. Males exclusively demonstrate parenting, while also expressing territorial aggression and courtship. In a semi-natural laboratory setting, wild-caught fish can be kept under conditions that mimic natural social groups (Reavis and Grober, 1999). The male, as the dominant individual, establishes a territory and actively defends his nest, a PVC tube. Males do jerk swims as courtship displays, while females solicit males through postural displays of their gravid abdomen (Pradhan and Grober, unpublished results). During spawning, females lay adhesive eggs on the inner surface of the nest, and the male fertilizes and cares for them until they hatch. Males exhibit high rates of parenting for multiple overlapping broods, including vigorous rubbing and aeration of eggs by fanning. Parenting males also display aggressively towards egg predators, including conspecific females, and leave the nest unguarded for only a few seconds at a time to feed or interact with females. We examined whether neurosteroids regulate parenting in *L. dalli* based on the following observations. First, parenting males exude high levels of KT in water compared to males not actively parenting and to females (Rodgers et al., 2006). Second, there are no sex differences between females and non-parenting males in levels of systemic, brain, and gonad KT (Lorenzi et al., 2012; Lorenzi et al., 2008). Third, brain KT levels are equal to, or higher than gonad KT, suggesting the brain is an important *de novo* producer of KT (Lorenzi et al., 2012; Pradhan et al., 2014). Finally, brain and gonadal KT levels are not correlated (Lorenzi et al., 2012).

We conducted a series of four experiments to test the acute neuroendocrine regulation of parenting in *L. dalli* through the use of carbenoxolone (CBX), a potent 11β-HSD inhibitor. The activity of 11β-HSD has been well studied in glucocorticoid conversion pathways in mammalian
tissues (Jellinck et al., 1993); however, its role in KT synthesis has received little attention. In the first experiment, we tested whether CBX regulates 11β-HSD by inhibiting the conversion of 11β-OHT to KT in brain and testis tissues. In the second experiment, to examine whether in vivo systemic CBX treatment reduces systemic KT levels, we intraperitoneally (IP) implanted breeding male L. dalli with either CBX or beeswax (vehicle). We predicted that exogenous CBX treatment should inhibit the synthesis of KT, resulting in decreased levels of KT exuded in water. In the third experiment, we tested the hypothesis that neurosteroids directly regulate parenting by manipulating brain steroid hormone levels via intracerebroventricular (ICV) injection of CBX or vehicle into parenting males. The effects of CBX on parenting could be due to direct effects on glucocorticoids (elevated cortisol) and/or androgens (decreased KT) (Knapp, 2003; Perry and Grober, 2003) (Figure 4.1). Consequently, we had two predictions regarding the mechanism(s) of CBX action. If reduced parenting was due to CBX-induced decreases in KT synthesis, then delivery of KT along with CBX should rescue parenting. However, if reduced parenting was due to elevated glucocorticoids, then cortisol delivery alone should also reduce parenting. Finally, in the fourth experiment we tested behavioral and endocrine responses of females and consequent effects on male reproductive success in response to a social context that resulted from Experiment 3 (reduced male attendance in the nest). Bluebanded gobies can undergo socially controlled sex change (Reavis and Grober, 1999), and as a result, our manipulations provided females with the opportunity to respond to changes in social context by exhibiting male-typical behavior.
4.3 Methods

4.3.1 General methods

All fish for these experiments were collected off the coast of Catalina Island, California during the months of June – July of 2009, 2011, and 2012 by SCUBA diving and using hand nets (permit numbers SC-10676 and SC-11879). After capture, the fish were kept in 2 L plastic Nalgene bottles, brought to the boat, and then placed in a large bucket for transport to a laboratory at the Wrigley Institute for Environmental Studies. Fish were housed in 60 x 94 cm\(^2\) aquaria with continuous seawater and exposed to natural ambient light cycles. Animals were fed twice daily, at 8:00 and 16:00 h with frozen brine shrimp. Most procedures involving direct handling, such as measurement of standard length (SL), surgeries, and necropsies, were conducted under a dissecting microscope. All chemicals used in these experiments were purchased from Sigma-Aldrich, unless specified. All data were analyzed using Prism 4.0a for Macintosh. Data were transformed where necessary and presented as mean ± s.e.m; all statistical tests were two-tailed (unless specified), and alpha was set at 0.05. All solvents used in this experiment were LCMS grade.

4.3.2 Experiment 1: effect of CBX on in vitro 11β-HSD activity

To measure the conversion of OHT to KT, we used an in vitro approach modified from previous studies (Pradhan et al., 2010; Rasheeda et al., 2010). Upon euthanization with tricaine methanosaltrate (MS-222; 1.0 mg/100 mL water), brains and gonads from adult male *L. dalli* (n=3) were removed within 5 min, rapidly frozen on dry ice, and stored at -80°C until biochemical assays. Each tissue type was pooled and homogenized with a hand-held homogenizer, using bursts, in ice-cold sucrose-Tris buffer (250 mM, pH 7.4). Samples were then centrifuged using a 5804 R Eppendorf Centrifuge at 1000 x g for 30 min, at 4°C to obtain
supernatants. Samples were divided into aliquots with the following treatments: buffer and drug vehicle only (negative control), tissue supernatants only, and tissue supernatants with 50 µM CBX. The samples were then incubated by shaking (60 rpm, New Brunswick shaker) at 25°C for 60 min, with 0.5 mM OHT (substrate for 11β-HSD) and 0.5 mM NAD⁺ (co-substrate for 11β-HSD) for a final reaction volume of 100 µL. Reactions were terminated by immersing the tubes in a methanol dry ice bath, after which 0.1 µM corticosterone (B, internal standard for liquid chromatography mass spectrometry (LC-MS/MS) analyses) was added to each sample and vortexed for 5 min. Prior to the extraction, 50 µL of methanol was added to all samples and vortexed thoroughly, after which water (400 µL) was added to each sample. Steroids were extracted from samples using C18 columns (Sepak 1cc, 50 mg sorbent, Waters). Columns were primed with 1 mL methanol (2x) and equilibrated with 1 mL water (2x). After the diluted tissue samples ran, the column was purged with 1 mL water (2x). Samples were then eluted with 250 µL methanol (2x), into vials (waters) and dried with a gentle stream of nitrogen in a water bath at 37°C. Samples were then resuspended in 50 µL 50% methanol (diluted with water), vortexed thoroughly, and stored at -20°C until LCMS analysis. We used a Triple Quadrupole LC-MS/MS mass spectrometer, Agilent 1200 (Agilent Technologies Inc, USA), equipped with a binary pump, auto sampler, and a Gemini 3µ 110A C18 column (Phenomenex). Two injections were taken from each sample for analysis, and a blank sample was run between each fish sample for subtraction of the background. To obtain separation of steroids, the solvents (Solvent A, water and Solvent B, acetonitrile) were modulated using isocratic steps over the course of 14 min. From 0-2 min, 50% Solvent A, 2.1-5 min 30% Solvent A, 5.1-9 min 0% Solvent A, and 9.1 to 14 min, 50% Solvent A. Throughout each run, the pressure was carefully monitored under a flow rate of 600 µL min⁻¹ using water (solvent A) and acetonitrile (solvent B), both containing 0.1%
formic acid. Retention time was based on the standards and relative retention time of the internal standard for each sample. Analyst 1.5.1 software was used for data quantitation (calculation of peak area ratio relative to the internal standard) and data were analyzed using one-way ANOVA.

4.3.3 **Experiment 2: Effect of systemic CBX implants on KT exuded into water**

4.3.3.1 **Surgery**

Fish were anesthetized with MS-222 (0.5 mg/100 mL water) to determine standard length (SL). Fish were housed in pairs, consisting of one male (SL= 31.16 ± 0.54) and one female (SL= 26.24 ± 0.42). These morphological differences ensure that fish established a robust linear hierarchy within 5 d (Reavis and Grober, 1999). Each pair was provided with a PVC tube (7.62 cm in length, 3 cm in diameter) that served as the nest. After 5 d, each male was implanted IP with either beeswax (Natural Cosmetics) only (vehicle, n=5) or beeswax + CBX (n=4) using previously described procedures (Pradhan et al., 2014). Implants were prepared by heat sterilizing beeswax and molding into small pellets under a microscope. Carbenoxolone was added to beeswax in 1:3 ratio, and each pellet was sterilized again by immersion in 100% ethanol for 1 s and dried. In brief, for the surgery, a small ventral incision was made anterolateral to the vent, the pellet was inserted, the skin was sealed with a cyanoacrylate adhesive (Henkel Loctite), and Stress Coat (API Pharmaceuticals) was applied over the incision site. The fish was kept moist with seawater during the surgery. On average, the surgery took 432 ± 51.62 s for vehicle fish and 382.5 ± 64.08 s for CBX fish (t=0.6096, p=0.54). Post-operatively, each fish was resuscitated and transferred to a clean cup of water for collection of exuded steroid hormone, and then the same pairs of fish were placed back into their tanks.
4.3.3.2 Collection and extraction of steroids from water

Steroids exuded into water via excretion through gills or urination is a good representation of system-wide steroids (Gabor and Contreras, 2012; Lorenzi et al., 2008). This non-invasive method of collecting steroids is particularly appropriate and useful for small fish due to the difficulty and/or impossibility of collecting sufficient plasma (Scott et al., 2001). Samples of KT exuded in water were also collected for 1h, 1 d, and 4 d post treatment. Previously validated procedures (using Sepak 2 cc, 200 mg columns, Waters) were used for extraction of steroid hormones from water (Lorenzi et al., 2008)

To collect steroids exuded in water, each fish was placed for 1 h in a clean, sterilized cup filled with 100 mL clean seawater that was kept cool via immersion in a water table with 5.08 cm of flowing water (Lorenzi et al., 2008). Steroids were extracted by passing the water through plastic tubing attached to Waters C18 columns (Sepak 2cc, 200 mg sorbent, Waters) and into a vacuum-assisted 12-port manifold. Prior to sample delivery, columns were primed with HPLC grade methanol (2 mL x 2), followed by equilibration with dH2O (2 mL x 2), after which both the ends of each C18 columns were wrapped in parafilm and stored at -20°C prior to being shipped to Georgia State University (GSU). After shipment, the columns were again stored at -20°C until extraction. Steroid extraction from columns was completed as per (Lorenzi et al., 2008). Briefly, the columns were thawed for 30 min on wet ice, equilibrated with dH2O (2 mL x2). The steroids were then eluted with methanol (HPLC grade; 2 mL x2), collected in test tubes and dried under nitrogen at 37°C. The dried samples were resuspended in 17.5 μL ethanol, vortexed for 1 min, and diluted into 332.5 μL enzymeimmuno assay (EIA buffer). The tubes were shaken for 1 h, parafilmed, and stored at -20°C until EIA.
4.3.3.3 Enzymeimmunoassays

Re-suspended samples were shaken on a multi-tube vortexer for 1 h before beginning the EIA procedure. All samples were assayed in duplicate, following the protocol in the package insert, with modifications and validations as noted in (Lorenzi et al., 2012). Briefly, after addition of a mouse anti-rabbit IgG monoclonal antibody and specific anti-11-ketotestosterone (KT) or anti-cortisol enzymatic tracer (KT- or cortisol-acetylcholine esterase conjugate), each plate was incubated for 18 h at 4°C on an orbital shaker. Details on the cross-reactivity of the assay and quality of standard curves can be found on the supplier’s (Cayman) website (https://www.caymanchem.com/app/template/Product.vm/catalog/582751). Following the addition of Ellman’s Reagent, plates were read using a Victor X Multilabel Plate Reader (Perkin Elmer). As per the recommendations of the manufacturer, absorbance wavelength was set between 410 and 415 nm, readings were taken after 60, 75, 90, and 105 min of incubation, and data from the time-point with the best standard curve ($R^2 > 0.9$) were used for all further analyses. All systemic steroid data are presented as pg sample$^{-1}$ (pg mL$^{-1}$ multiplied by the volume in which the dried sample was initially was suspended) and adjusted for the dilution, which was the amount of EIA buffer used to re-suspend the sample). Data were analyzed using two-way repeated measures ANOVA, with time as the within subjects factor and treatment as the between subjects factor.

4.3.4 Experiment 3: effect of ICV CBX on male parenting

This is the first study to inject an enzyme-inhibiting drug into the brain of a fish to investigate effects on social behavior, but our methods of ICV injection and recovery were modified from a previous injection study (Solomon-Lane and Grober, 2012). Therefore, in a pilot experiment, we investigated the appropriateness and efficacy of ICV CBX dose by
examining recovery from injections, basic locomotion, social interactions and concentration of KT and cortisol (Steraloids Inc.) to be injected (see below).

4.3.4.1 Pilot study

During mid-breeding season, social groups of *L. dalli*, each consisting of one male (SL= 36.17 ± 0.55 mm) and two size-mismatched females were constructed (alpha SL= 31.15 ± 0.20 mm; beta SL= 27.31 ± 1.34 mm). Over a period of 3 weeks, daily egg checks were conducted to ensure that males were experienced at spawning and caring for eggs, and had at least 2 clutches of eggs during the breeding season. Prior to injections, baseline behavior was measured (see below). The vehicle (n=7) used was sterilized phosphate buffer (0.1 M), the amount of CBX used was 1265 ng/brain (n=7), and each injection volume was 50.6 nL. The procedure described in the *ICV injections* section below was followed, except that each fish was out of the water for 2 min during the injection, after which it was immediately placed in a cup of clean seawater to monitor recovery and the fish was re-united with the females. Parenting decreased significantly in males treated with CBX (number of parenting bouts: Vehicle= 49.56 ± 13.12; CBX= 3.88 ± 3.73; t15=3.17, p=0.0063) and after exactly 60 min, the males from the groups treated with vehicle and CBX were removed and euthanized with MS-222 kept on ice. It took an average of 6.75 ± 0.52 min to remove brains of vehicle injected animals and 6.25 ± 0.65 min for the CBX injected animals (t12=0.5991, p>0.05) which were then immediately frozen on dry ice. At the end of the field season, all brains were shipped to Atlanta at -80°C.

4.3.4.2 ICV injections

The Nanoject II Auto-Nanoliter Injector (Drummond Scientific Company, Broomall, PA, USA) was used and all injections were conducted between 9:00 and 12:00 h. No more than six injections were done on a particular day, and the experiment was conducted over a period of 5 d.
To prepare the KT and cortisol solutions, 0.1% ethanol (prepared in phosphate buffer) was used and hence the same volume of ethanol was added to the vehicle stock. Each vial containing the solutions to be injected was kept on ice during the experiment and thoroughly vortexed before use, loaded immediately into the needle, and only one injection was performed with that loaded needle. Each fish was out of the water for 2 min during the injection, after which it was immediately placed in a cup of clean seawater to monitor recovery. Observers conducting the post-injection physiological measurements were blind to the fish’s treatment. The fish was undisturbed for the first 10 min and care was taken to minimize vibrations produced by human movement in the room. Fish ventilation rate can be measured by counting the opening and closing of the operculum. After 10 min, manual resuscitation, using a plastic Pasteur pipette, was conducted if the fish did not initiate ventilation independently. Time taken to initiate ventilation and time taken to regain equilibrium were recorded (Figure 4.2). In addition, opercular movement was recorded every 30 s until the male regained equilibrium. One minute after the male regained equilibrium, a dime was dropped in the cup from 17.8 cm above to record the startle response and locomotion. This procedure generated a swift and immediate startle response in non-injected individuals, and hence, was used to assess basic sensorimotor capacities.

4.3.4.3 Collection and extraction of steroids from tissue

For experiments where tissues were to be collected, animals were sacrificed within one minute of disturbance of the group by euthanizing with MS-222 (1.0 mg/100 mL water). Brains were rapidly removed and frozen on dry ice, after which gonads were removed and frozen. All tissue samples were stored at -80°C until shipment to GSU on dry ice, and stored again at -80°C until extraction. On the Day 1 of the tissue extraction procedure, each tissue was weighed (wet
weight) on a Metler Toledo electronic balance and homogenized in 350 µL ice-cold phosphate buffer (0.1 M, pH = 7.4) and 1500 µL HPLC grade methanol was added to the mixture and vortexed for 1 min and placed back on ice. All samples were then shaken in a multi-tube vortex for 1 h at room temperature. The homogenates were then placed at 4°C overnight. The following day, samples were again shaken in a multi-tube vortex for 1 h at room temperature. The samples were then centrifuged for 10 min at 1000 g at 4°C and diluted with 9 mL of ddH2O. C18 columns (Sepak 6cc, 500 mg sorbent, Waters) were primed with 5 mL HPLC grade methanol (2x) and equilibrated with 5 mL ddH2O. The samples were then loaded to the column, followed by another 5 mL ddH2O (2x). For the final elution step, 5 mL HPLC grade methanol was used, after which the procedure was the same as described above in the “Collection and extraction of steroids from water” section.

4.3.4.4 Main experiment

During mid-breeding season, social groups of *L. dalli*, each consisting of one male (SL= 41.44 ± 0.53 mm) and two size-mismatched females (SL: alpha=35.13 ± 0.29 mm; beta= 30.72 ± 0.24 mm) were constructed. The male was the largest and dominant member of the group and was at least 3 mm larger than the biggest female (alpha, subordinate only to the male); the beta female (most subordinate) was at least 3 mm smaller than the alpha female. Each group was provided with an acetate-lined PVC tube (7.62 cm in length, 3 cm in diameter) that served as the nest. Over a period of 4 weeks, daily checks were conducted to ensure that males were experienced at parenting (2-4 clutches). On the day before injections, groups were chosen for use if their males were actively parenting in the nest. All the fish from the selected group, along with their acetate-lined PVC tube containing eggs, were placed in a different experimental tank that allowed for more convenient behavioral observations and injections. On the day of the injections, observers,
blind to the particular treatment, recorded pre-injection behavior (see below). Within seconds after the baseline observations, the male was removed and anesthetized. Alpha and beta females were placed in separate cups during male injections and recovery, to ensure that they did not have access to the unprotected eggs and that dominant females did not initiate behavioral and physiological transformations associated with sex change in the absence of the male. Each injection procedure took 1 min, after which, the recovery was measured (Figure 4.2), as explained above. This experiment consisted of the following treatment groups: (i) vehicle (n=7), (ii) CBX (1265 ng brain⁻¹, n=9), (iii) CBX+KT (1265 ng CBX + 0.50 ng KT brain⁻¹; n=9), and (iv) cortisol (0.05 ng brain⁻¹; n=9). The CBX dose was scaled to match the dose used for an ICV study in rats (Jellinck et al., 1993) and the recovery of fish in our pilot studies. The scaled KT and cortisol doses were based on the highest brain levels of these steroid hormones following CBX injections in the pilot experiment. Briefly, in that study, we found that 60 min following ICV injection, brain KT levels did not change significantly (vehicle=195.80 ± 177.40 pg mg⁻¹ tissue, CBX= 20.25 ± 5.58 pg mg⁻¹ tissue; t₁₂=1.11, p=0.29), however, cortisol levels increased significantly (vehicle= 13.71 ± 1.65 pg mg⁻¹ tissue, CBX= 32.53 ± 4.67 pg mg⁻¹ tissue; t₁₃=3.10, p=0.008). Overall, the ratio of KT to cortisol (KT:cortisol) decreased significantly (vehicle= 17.97 ± 16.53 pg mg⁻¹ tissue, CBX= 0.59 ± 0.16 pg mg⁻¹ tissue; t₁₂=2.33, p= 0.038). These data were converted to the units required for ICV injection for 5 mg tissue, which is the average weight of an L. dalli brain (unpublished data).

After recovery, the male was returned to his tank, and females were re-introduced 1 min later. Latency for the male to enter the nest was recorded over a period of 61 min. Immediately after the group was reunited, post-injection rates of social behavior were measured in 10 min intervals for the first 30 min and then from 50-60 min. Time between the injection and start of
behavioral observations did not differ significantly among the four groups (Kruskal-Wallis statistic, $H=0.136$, $p=0.98$). Before and after ICV injections, we quantified agonistic behaviors, which consisted of approaches (when one fish came within two body lengths of another fish) and displacements (if the approached fish responded by swimming away). Rates of agonistic behavior were calculated by dividing the number of occurrences of each type of behavior by 10 min, which was the length of each observation period. Parenting was quantified by counting the number of bouts of egg care, including vigorous rubbing and fanning of eggs inside the nest using fins. Two independent bouts were separated by at least 2 s, and each bout could last from 1 to several seconds. Previous observations showed that measuring the frequency of fanning bouts was a good predictor of the duration of bouts of parenting ($r^2=0.52$, $p<0.0001$). For logistical reasons, it was not possible to accurately measure bout length in this study, so we only recorded the number of parenting bouts. Before injections, males from all groups spent the majority of their time in the nest (91.80 ± 2.17 %); however, there was no relationship between time in the nest and number of parenting bouts ($r^2=0.02$, $p=0.46$). By dividing the number of parenting bouts by the amount of time spent in the nest, we are able to distinguish high parenting males who did not spend much time in the nest from those who spent a lot of time in the nest, but did not exhibit high rates of parenting. From our pilot studies, we found that behavior changes over time, after males are re-united with the social group following recovery from the ICV injection. To control for this, to focus on rapid effects on behavior, and to avoid pseudoreplication, the time spent in the net and parenting bouts were analyzed for the 10-20 min sample only. We did not choose the first 10 min, because males were still recovering and rates of all social behaviors were reduced in all treatment groups. Of the remaining groups, the 10-20 min sample was closest to the time of injection, but rates of agonistic interactions were returning
to pre-injection levels. One-way ANOVA was used to compare the treatment groups for latency to enter nest, time in nest from 10-20 min, and number of parenting bouts from 10-20 min, followed by post-hoc Tukey Multiple Comparison tests. Additionally, rate of approaches, rate of displacements, number of parenting bouts, and number of parenting bouts/time in nest were analyzed using a series of two-way repeated measures ANOVA with treatment as the between subjects factor and time as the within subjects factor, followed by post-hoc Bonferroni tests.

4.3.5 Experiment 4: effect of context on male reproductive success, female behavior, and tissue KT

The changes in male behavior caused by some ICV injections (1 vehicle, 7 CBX, and 4 cortisol) in Experiment 3 created a rare social context that permitted females to enter the nest and consume eggs and/or exhibit parenting, even while the male was in close proximity. In other (control) groups, male behavior inside the nest inhibited females from entering the nest. For females that interacted with the eggs, we recorded the number of bouts of egg eating and parenting using methods similar to those of Experiment 3. When all animals were removed from the home tank for male injections, the acetate was removed from the PVC tube, placed in a frame, immediately photographed (Canon SX150 IS) and replaced in the tube. This process was repeated again at the end of the 60 min post-injection behavioral observation period. All the photographs were uploaded to a Macintosh computer, and eggs were quantified using ImageJ. Number of eggs is a direct measure of male reproductive success and we predicted the number of eggs to decrease for two reasons. First, females do not generally lay eggs in the absence of a male, so we did not expect the number of eggs to increase. Second, in our pilot experiment we observed females eating eggs in the absence of a male. Hence we used a one-tailed t-test to compare the percent of eggs lost between the vehicle- and CBX-treated groups. At the end of the
60 min behavior observations, females were euthanized, and their brain and gonad tissues were immediately collected, frozen on dry ice, and maintained at -80°C until assayed. Steroid hormone extractions were conducted using previously described techniques (Lorenzi et al., 2012; Newman et al., 2008) (see supplemental material for details), and differences in tissue KT of parenting versus non-parenting alphas females were analyzed using unpaired t-tests.

### 4.4 Results

#### 4.4.1 Experiment 1: effect of CBX on in vitro 11β-HSD activity

After 60 min of incubation, the addition of CBX reduced the KT:B peak area ratio (Figure 4.3) by 90% in brain (one-way ANOVA, $F_{2,5}=147.1$, $p=0.001$) and 100% in testes (one-way ANOVA $F_{2,5}=190.4$, $p=0.0007$).

#### 4.4.2 Experiment 2: effect of CBX implants on KT exuded in water

Levels of KT exuded in water were transiently affected following IP CBX implants in males (Figure 4.4). Two-way repeated measures ANOVA revealed that there was a significant main effect of time ($F_{2,7}=0.17$, $p=0.018$), no main effect of treatment ($F_{1,7}=0.17$, $p=0.69$), and no interaction between time and treatment ($F_{2,7}=1.43$, $p=0.27$). *Post-hoc* Bonferonni tests revealed that 1 d after implanting fish with CBX, KT levels were reduced by 48% compared to 1 h after treatment ($t=2.80$, $p<0.05$), and then significantly increased 4 d after treatment (1 d vs. 4 d, $t=2.68$, $p<0.05$).

#### 4.4.3 Experiment 3: effect of ICV CBX on male parenting

Following ICV injections, one-way ANOVA determined that there was a significant effect of treatment on males’ latency to enter the nest (Figure 4.5A, $F_{3,30}=3.74$, $p=0.02$), time
spent in the nest (Figure 4.5B, F_{3,30}=4.28, p=0.013), and number of parenting bouts (Figure 4.5C, F_{3,30}=6.67, p=0.001). Compared to vehicle-injected males, males injected with CBX took longer to enter the nest (q=4.59, p<0.05), and during the 10-20 min sample period they spent less time in the nest (q=4.90, p<0.01) and demonstrated much less parenting (q=5.99, p<0.01).

As predicted, males injected with a physiological dose of KT along with CBX did not differ significantly from the vehicle in latency to enter the nest or time in the nest (both p>0.05). Most importantly, in the 10-20 min sample, KT rescued the effects of CBX on number of parenting bouts (Figure 4.5C, CBX vs. CBX+KT, q=4.57, p<0.05). Compared to vehicle, ICV cortisol had no effect on males’ latency to enter nest, time spent in nest, and number of parenting bouts (all p>0.05).

More detailed analyses of behavior using time as a repeated measure following injection (in 10 min intervals) revealed that the effects of treatment were specific to parenting and did not affect other social behaviors (Figure 4.6). For rates of agonistic interactions, there was no significant main effect of treatment for male approach rate (F_{3,30}=0.85, p=0.48) or displacement rate (F_{3,30}=0.78, p=0.37), but there was a significant main effect of time (approaches, F_{4,30}= 5.22, p=0.007; displacements, F_{4,30}= 8.94, p<0.0001). There was no interaction between treatment and time (approaches, F_{12,30}=0.60, p=0.84; displacements, F_{12,30}=0.80, p=0.65). For male parenting bouts, there was a significant main effect of treatment (F_{3,30}=5.38, p=0.004) and a main effect of time (F_{4,30}= 6.66, p<0.0001), but there was no interaction between treatment and time (F_{12,30}=0.52, p=0.90). For male parenting bouts/time in nest, all patterns were the same, such that there was a significant main effect of treatment (F_{3,30}=7.10, p=0.001) and a main effect of time (F_{4,30}= 9.56, p<0.0001), but there was no interaction between treatment and time (F_{12,30}=1.10, p=0.37). While overall rates of all social behaviors in other treatment groups
increased to pre-injection levels as function of time, parenting behavior in 89% of CBX-treated males did not recover to baseline within 60 min of ICV injection (Figure 4.6C).

**4.4.4 Experiment 4: effect of context on male reproductive success, female behavior, and tissue KT**

Manipulation of some males (1 vehicle, 7 CBX, and 4 cortisol) produced a social context that permitted females to opportunistically enter the nest tube without the male. In the first 30 min, alpha females (44%) and beta females (15%) that entered the nest exhibited egg consumption (Figure 4.7A,B). The highest consumption was observed between 10-20 min (p<0.05), reducing number of eggs by 15.76 ± 5.74% (t12=2.03 p=0.03). A subset of the alpha females (34%) that entered the nest while the male was in close proximity, but not in the nest, also demonstrated low and variable levels of male-typical parenting behavior at some point during the 60 min post-treatment observation period. Compared to alpha females that did not exhibit parenting, those that parented had significantly elevated brain KT (p<0.01; Figure 4.7C) and reduced ovarian KT (p<0.05; Figure 4.7D). In alpha females demonstrating parenting, brain KT:cortisol was 0.03 ± 0.01. Although female parenting was exhibited at a very low frequency compared to male parenting, this association is intriguing because this effect was specific to KT and not other steroid hormones (T, cortisol, E₂; all p>0.05, data not shown).

**4.5 Discussion**

We demonstrate that exogenous manipulation of a single steroidogenic enzyme in the brain, at the site of both its synthesis and action, can rapidly regulate parenting behavior and consequently reduce male reproductive success. We provide the first direct evidence that neural synthesis of KT is a pivotal mechanism for regulation of male parenting and that cortisol does
not decrease parenting in a species of teleost fish. We also show that brain KT is elevated in females that display parenting, a male-typical phenotype. We speculate that rapid modulation of local steroidogenesis allows for regulation of dynamic changes in behavior in an environment that requires an organism to successfully coordinate multiple activities to enhance fitness (Pradhan and Soma, 2012; Remage-Healey et al., 2008), including interaction with conspecifics, territory defense, foraging, and parenting. Our data indicate that neural synthesis of steroid hormones can be just as important for regulation of reproductive behavior as other sources of steroid hormones (e.g., gonads and inter-renal tissue (Schmidt et al., 2008)).

4.5.1 Effect of CBX on steroid hormone conversion pathways

Like other steroidogenic enzymes, 11β-HSD participates in the conversion of more than one steroid hormone (Figure 4.1). As a result, changes in 11β-HSD activity can potentially affect hormones in multiple pathways (Knapp, 2003). Previous studies have reported that CBX dramatically inhibits 11β-HSD activity in deactivation pathways for corticosterone (Jellinck et al., 1993) and cortisol (Webb et al., 2008). However, CBX has not been previously studied in the regulation of KT synthesis. We showed that CBX inhibits 11β-HSD activity and thus the production of KT in brain and testes (Figure 4.3). Our study demonstrates that it is possible to pharmacologically inhibit KT production and highlights the utility of CBX as a powerful tool for studies of the phenotypic effects of KT in a variety of species. We also demonstrated that IP CBX implants in males produced a transient decline in KT exuded in water, such that KT levels were reduced after 1 d, but recovered to control levels after 4 d (Figure 4.4). A previous study showed that following IP injections, CBX was not detectable in the cerebrospinal fluid of rodents, although it was detectable in plasma (Leshchenko et al., 2006). The authors concluded that CBX does not penetrate the blood brain barrier, and they recommend ICV injections to
induce brain effects of CBX (Leshchenko et al., 2006). If CBX similarly does not cross the blood-brain barrier in fish (Castejon, 1983), our data suggest that lower levels of KT 1 d after IP CBX implants are due to a reduction in peripheral KT synthesis. Finally, ICV CBX delivery allowed us to manipulate neurosteroids. Our pilot studies showed that whole brain KT:cortisol decreased significantly in CBX treated males. This approach allowed us to utilize a local manipulation to investigate the role of neurosteroids in regulating parenting.

4.5.2 Neurosteroidal regulation of parenting behavior

Within only 20 min, ICV CBX transiently eliminated parenting behavior, but not other social behavior, suggesting an enzymatic mechanism for rapid neurosteroidal regulation of parenting. The rapid and specific effects of CBX on male parenting are likely due to its inhibitory effects on neural 11β-HSD activity, which could have direct effects on glucocorticoids and/or androgens (Perry and Grober, 2003) and result in a local decrease in KT and/or elevation in cortisol. Consistent with our proposed mechanism, elevating KT levels rescued parenting when paired with CBX, while cortisol alone did not affect parenting.

By manipulating brain 11β-HSD activity and relative concentrations of KT and/or cortisol, we demonstrate that these steroid hormones do not decrease parenting, but rather can promote parenting. Causative relationships between changes in levels of systemic steroid hormones and reproductive phase-dependent changes in behavior have not been investigated extensively. In many bird species, systemic T levels decline in males at the onset of parenting (Ketterson and Nolan, 1992; Lynn and Wingfield, 2008; Wingfield et al., 2001). In a paternal anuran, plasma androgen levels decline between the sexually active stage and parenting stage, and levels then remain low throughout all stages of parenting (Townsend and H., 1987). In fish, the pattern of androgen involvement in parenting is less straightforward. In male sticklebacks,
circulating KT levels correlate with maturation of eggs: KT is high during courtship but lower during parenting. Systemic androgen treatment, however, does not decrease parenting rates (Páll et al., 2002a, b). In the peacock blenny, brood size is positively correlated with both plasma T and KT levels (Ros et al., 2004). However, in bluegills, KT mediates a tradeoff between aggressive and nurturing components of paternal care (Rodgers et al., 2013). In some mammals, circulating T is elevated during parenting (Townsend, 1987; Trainor and Marler, 2001), but this is associated with increased brain aromatization of T to E$_2$ (Trainor et al., 2003). In _L. dalli_, circulating levels of KT in parenting males are higher compared to females and non-parenting males (Rodgers et al., 2006). In many vertebrates, the role of androgens in the regulation of parenting remains elusive because the correlative patterns seen in most species may be a consequence of studying systemic steroid hormones and this might not reflect their action in the brain. This could be resolved by examining local steroid hormone and receptor levels within specific target tissues. This experiment is the first to provide a mechanism by which locally synthesized neural KT regulates parenting behavior.

The role of circulating glucocorticoids in regulating various aspects of reproduction and life history tradeoffs has been widely studied due to the general interest in the harmful impacts of chronic stress. Here, neural cortisol treatment reduced parenting in 55% of the males, but overall there were no significant effects. From the literature, the role of systemic cortisol in regulating parenting in fish is not resolved (Magee et al., 2006), but long-term cortisol treatment may increase the rate of nest desertion (O'Connor et al., 2011). Our data support the hypothesis that short-term ICV treatment with glucocorticoids does not reduce parenting. It is possible that glucocorticoids may support allocation of energetic reserves towards caring for the present clutch to maximize reproductive success (O'connor et al., 2012).
4.5.3 Role of neural steroidogenic enzymes in regulating transitions between behavioral states

The integration of environmental stimuli (e.g. social factors, food availability or stress), endocrine cues from extra-neural sources, and the local ratio of steroid hormones within the brain can generate a highly context specific means of rapidly gating behavioral output. Male *L. dalli* maintain the highest rank in the social group, while interacting with conspecifics in a variety of ways, including territory defense, courtship, spawning, and parenting. Moreover, these behavioral states are dynamic and fluctuate within seconds. We propose a neurosteroidogenic mechanism for the inhibition and release of these behaviors via temporally (rapid) and spatially (local) controlled changes in the activity of a single enzyme. We showed that decreased 11β-HSD activity can potentially depress brain KT and simultaneously increase brain cortisol, thereby providing a means to rapidly modify the local ratio of these two steroid hormones. Consistent with this idea, female *L. dalli* generally do not provide parental care, and brain KT:cortisol is two orders of magnitude lower in females than in parenting males (see below). However, changes in conversion of either of these steroid hormones can independently regulate parenting. Our data do not provide evidence for local cortisol regulation of parenting on a short time-scale. We suggest that brain 11β-HSD activity functions as an enzymatic switch to shift males between behavioral states via changes in local KT levels, such that high KT increases parenting and low KT reduces parenting.

In addition, the reproductive plasticity of *L. dalli* permit bidirectional adult sex change – a process that involves multiple changes throughout the body axis (Reavis and Grober, 1999). Initiation of sex change involves rapid increases in rates of aggressive behavior in the dominant female undergoing sex change (Reavis and Grober, 1999). This is accompanied by rapid
reduction in brain aromatase activity, thus providing a mechanism for a local reduction in brain E\textsubscript{2} (Black et al., 2005). Brain KT also increases in females during sex change and this could occur via an increase in brain 11\beta-HSD activity (Black et al., 2005; Lorenzi et al., 2012). Here, in the absence of males occupying the nest and caring for eggs, opportunistic females rapidly monopolized these resources. In addition to engaging in agonistic interactions outside the nest, several alpha and beta females gained access to the nest, leading to consumption of 15\% of the eggs within 30 min. Alpha females from 12 groups also exhibited substantial rates of parenting. These females were likely demonstrating the onset of behavioral sex change in the absence of male domination and had high brain KT and low ovarian KT relative to females that did not care for eggs. These data provide evidence that females that exhibit parenting (albeit for a short amount of time) also have high KT, similar to parenting males (Rodgers et al., 2006). A rapid increase in brain 11\beta-HSD activity can be a mechanism for elevated brain KT, but it is also possible that these females had naturally high brain KT prior to the social manipulation. Female L. dalli appear to be extremely sensitive to changes in social context (Pradhan et al., 2014), and this behavioral plasticity ultimately has the potential to increase fitness. Our data provide a clear demonstration of social dynamics having differential effects on local steroid hormone levels and behavioral output. Our data also demonstrate that elevated neural KT is associated with parenting in both males and females.

4.5.4 Conclusions

Our results support the hypothesis, posited by Perry and Grober (Perry and Grober, 2003), that regulation of 11\beta-HSD can play a key role in the induction of major life history transitions. This local mechanism of neural steroidogenesis could be advantageous for the speed of action, thus reducing the inappropriate expression of behavior in unpredictable environments.
Although our study focused on the role of neural 11β-HSD in regulating parenting, we suggest that this mechanism of local regulation could have broad importance in the context of other enzymes, such as aromatase, 5β-reductase, and 3β-hydroxysteroid dehydrogenase and other classes of social behavior (Ball and Balthazart, 2004; Black et al., 2005; Pradhan et al., 2010; Remage-Healey et al., 2008).

4.6 Acknowledgements

We thank Cory Grober, Captain Jack, Pierre Naude, Kevin Thonkulpitak, Hannah Shin, Jason Crutcher, and David Sinkiewicz for help with fieldwork, behavioral observations, and assays; Mary Karom, Drs. Siming Wang, Lifang Wang, Anne Murphy, and Walter Wilczynski for technical support; and the staff at USC Wrigley Institute for Environmental Studies for logistical assistance. We also thank Dr. Knapp and two anonymous referees for comments on earlier drafts of the manuscript. We are grateful for the grants from NSERC PGS D3, Sigma Xi, Brains & Behavior Program at Georgia State University and Georgia State University Dissertation Award to DSP, NSF (IOB – 0548567) to MSG, and NSF Doctoral Dissertation Improvement Grant (1210382) to MSG and DSP.

4.7 References


Figure 4.1: Simplified pathway of steroidogenesis in fish. Testosterone is converted to 11-Ketotestosterone (KT) via the sequential action of 11β-hydroxylase, which converts KT to 11β-hydroxytestosterone (11β-OHT), and 11β-hydroxysteroid dehydrogenase, which converts 11β-OHT to KT and cortisol to cortisone. Inhibition of 11β-HSD with carbenoxolone (solid grey ‘X’) can elevate cortisol and reduce KT (solid grey arrows) levels.
**Figure 4.2:** Effect of carbenoxolone (CBX) and 11β-hydroxytestosterone (OHT, an endogenous substrate) on 11β-hydroxysteroid dehydrogenase activity in adult male *L. dalli* (a) brain and (b) testes. Tissue supernatants were incubated *in vitro* at 25°C with 1 mM NAD⁺ as a co-substrate for 60 min. Controls were incubated with buffer + vehicle only. 11-Ketotestosterone (KT) peak area ratio was calculated by dividing area of KT peak to that of corticosterone (B), the internal LCMS standard (N=3 males per group); **p<0.01, ***p<0.001.
Figure 4.3: Effect of carbenoxolone (CBX) and 11β-hydroxytestosterone (OHT, an endogenous substrate) on 11β-hydroxysteroid dehydrogenase activity in adult male *L. dalli* (a) brain and (b) testes. Tissue supernatants were incubated *in vitro* at 25°C with 1 mM NAD⁺ as a co-substrate for 60 min. Controls were incubated with buffer + vehicle only. 11-Ketotestosterone (KT) peak area ratio was calculated by dividing area of KT peak to that of corticosterone (B), the internal LCMS standard (N=3 males per group); **p<0.01, ***p<0.001.
Figure 4.4: Effect of intraperitoneal (IP) implants of carbenoxolone (CBX), an 11β-hydroxysteroid dehydrogenase inhibitor, on 11-ketotestosterone (KT) exuded in water (systemic levels) 1 h, 1 d, and 4 d post-treatment of adult male *L. dalli*. Vehicle: n=5 and CBX: n=4; *p<0.05, **p<0.01
Figure 4.5: Effect of intracerebroventricular (ICV) injection of parenting male *L. dalli* on (a) latency to enter nest, (b) time spent in nest between 10-20 min, and (c) number of parenting bouts between 10-20 min. Vehicle: n=7; Carbenoxolone (CBX): n=9; CBX + 11-ketotestosterone (KT): n=9; Cortisol: n=9; *p<0.05, **p<0.01.
**Figure 4.6:** Effect of intracerebroventricular (ICV) injection of parenting male *L. dalli* on agonistic interactions and parenting behavior; (a) male approach rate, (b) male displacement rate, and (c) male parenting. Vehicle: n=7; Carbenoxolone (CBX): n=9; CBX + 11-ketotestosterone (KT): n=9; Cortisol: n=9; *p<0.05 **p<0.01.
**Figure 4.7:** Effect of ICV treatment of parenting males on female interaction with eggs (a)

Percentage of egg loss in groups with males treated with vehicle (n=7) or CBX (n=9) (b) Female egg eating bouts as a function of time relative to ICV injections of the male (c) Brain and (d) Ovarian KT levels in females who did not parent (n=22) and those who exhibited parenting (n=12); *p<0.05, **p<0.01.
5 MULTI-TASKING MALES AND SEX-ROLE REVERSED FEMALES IN HAREMIC BLUEBANDED GOBIES, *LYTHRYPNUS DALLI*

5.1 Abstract

While males typically compete for females, there are many species in which the sex ratio is skewed towards females and/or males invest heavily in reproductive effort. Those species exhibit reversed sex roles in sexual advertisement signals and intrasexual competition. Competing demands on male investment may also drive reproductive tradeoffs. Since variation in both male and female behavior might affect reproductive success, here, we investigated behavioral contributions of both sexes to male reproductive success in a haremic fish, the bluebanded goby, *Lythrypnus dalli*. In this species, the operational sex ratio is female-biased and males invest heavily in nest defense and offspring care, so consequently, females might compete for males. Males demonstrate requisite paternal care, maintain the highest social rank, defend their nest/territory, and court females. As a result of overlapping time demands, courtship and territorial defense could reduce the quality or quantity of parenting, and impact offspring survival. Over 3 weeks, we documented natural variation in egg numbers and periods when there were no eggs in the nest in groups consisting of one male and two size-mismatched females. We investigated whether the presence of eggs in the nest impact rates of courtship and nest care. Next, we examined if variation in reproductive success (quantified as number of eggs) was associated with the expression of specific reproductive behaviors. Male nest care, male courtship, and female courtship were not affected by egg presence. Overall, females courted at a higher rate than the male. Egg numbers were not associated with rates of male behavior such as nest care, approaches towards females, or courtship, but were associated with rates of female
courtship. More specifically, number of eyed eggs was associated with rates of courtship solicitations by the alpha female. Also consistent with predictions for a role reversed reproductive strategy, intra-sexual competition was exhibited in the form of alpha females interrupting courtship solicitations by beta females. Following an interruption, alpha females frequently courted males, and in those cases, the male approached the alpha female. Overall, these data provide evidence for sex role reversal in *L. dalli* and that other aspects of male social behavior do not interfere with expression of paternal care.

### 5.2 Introduction

Elaborate male sexual advertisement signals, male-male competition, and female choice are the hallmarks of sexual selection (Flamarique et al., 2013; Maclaren and Rowland, 2006; Darwin, 1871). However, in at least some representatives from diverse taxa, including insects (Salehialavi et al., 2011; Rutowski, 1984), birds (Voigt and Goymann, 2007; Griggio et al., 2008), reptiles (Swierk et al., 2013), amphibians (Bush and Bell, 1997), fish (Svensson et al., 2005), and primates (Van Belle et al., 2009), the typical sex roles are reversed and females court and compete for males. Male mate choice is especially important in polygamous social systems where the operational sex ratio is skewed towards females (Trivers, 1972). In many such cases, the male invests heavily in parenting in terms of energetic expenditure, susceptibility to predators, compensation for the lack of egg care by females and time away from courting potential mates (Westneat and Sargent, 1996). Thus, traits that allow a male to invest heavily in parenting may be under a greater selective pressure compared to courtship, status, or territory defense, which are central in many species to future reproductive opportunities. Thus, there may
be tradeoffs for males between current and future reproduction (Westneat and Sargent, 1996; Gross and Sargent, 1985).

Male reproductive costs can be reduced if females engage in behaviour to evoke appetitive responses (Beach, 1976; Swierk et al., 2013). Female courtship solicitation include attractive signals (e.g., postural displays or scent marking) and proceptive behaviours (indicating sexual readiness), which are essential stimuli that initiate male interaction, influence reproductive decisions and eventually lead to receptivity and copulation (Beach, 1976).

Bluebanded gobies, *Lythrypnus dalli*, are an ideal species to examine the contributions of sex-typical behaviour and social structure to reproductive success. These fish are polygamous and live in small haremic groups consisting of several females and one male. Little is known about the expression of sexually dimorphic behaviour in the field, but it can be rigorously studied in a semi-natural laboratory habitat. Once social groups are constructed, status resolution among the members can take up to 5 d (Reavis and Grober, 1999) and generally, the male occupies and defends the area around the nest. Agonistic interactions occur among all members of the group to establish and maintain a linear social hierarchy and female agonistic behaviours are associated with male reproductive success (Solomon-Lane et al., 2014). Male courtship behaviour includes jerk swims towards females of his choice, whom he leads into the nest for spawning. Demersal eggs are normally attached to the roof of the nest and in laboratory groups, eggs can be easily quantified (Solomon-Lane et al., 2014). The number of eggs in a nest is a common and effective measure of male reproductive success and as such is a useful proxy for male fitness (Solomon-Lane et al., 2014). In addition to providing exclusive care for overlapping broods, the male also defends the eggs from predators, including opportunistic females who will consume eggs.
Female *L. dalli* display solicitation behaviour similar to that documented in other fish species (Rowland et al., 2002), however this has not been described previously.

In *Study 1*, we examined basic parameters such as the length of time it takes for egg appearance in the nest after groups of wild-caught *L. dalli* are constructed in the laboratory and the developmental stages of eggs. Based on those results, we designed *Study 2*, in which we examined the relationship between a suite of sexually dimorphic reproductive behaviour and reproductive success in a species that exhibits behavioural traits consistent with sex role reversal. Using groups of *L. dalli* consisting of one male and two status-mismatched females, we documented natural variation in egg numbers, including periods without eggs in the nest. Based on the differences in egg laying documented across social groups, we first asked whether egg presence affects rates of male and female reproductive behaviour. We predicted that when eggs are absent from a nest, rates of male courtship will be higher and rates of male nest care will be lower. For females, we predicted that, as in other species (Knapp and Kovach, 1991; Marconatto and Bisazza, 1986), female courtship rates will be higher in the presence of eggs. Our second goal was to examine whether traits such as body size and sex-specific reproductive behaviors were associated with the number of eggs in the nest. Finally, to get a deeper understanding of how female rank modulated social interactions, we analyzed behavioural transitions among all fish within the group by constructing a transition matrix. Together, these data allowed us to determine whether *L. dalli* exhibit sex role reversal and examine tradeoffs in male reproductive behavior.
5.3 Methods

5.3.1 General procedures

The habitat of *L. dalli* ranges from the rocky reefs of Gulf of California, Mexico, to Morro Bay, California (Miller and Lea, 1972). All fish in the present study were collected from the reefs off the coast of Catalina Island, California by SCUBA diving and using hand nets (permit number SC-11879) in July 2011 and 2012. After capture, the fish were placed in 2 L plastic Nalgene bottles, brought to the boat, and placed in a large bucket for transport to the laboratory in the Wrigley Institute for Environmental Sciences. Fish were housed in a 60 x 94 cm² aquarium supplied with continuous seawater and exposed to natural ambient light cycle. The day after capture, fish were anesthetized with tricaine methanosulfate (MS-222; 0.5 mg / 100 mL H₂O) and placed under a dissecting microscope for morphological measurements. Standard length (SL) was measured using a vernier caliper. The external genitalia were imaged using a camera (Motic Images) attached to an Apple MacBook, and the specific pattern of blue bands was noted for each fish and helped with identifying individuals. Groups were constructed based on SL of fish, such that the male was the largest, and additional females differed in SL by at least 3 mm. These differences in size ensured rapid establishment of a linear hierarchy (Reavis and Grober, 1999) and easy identification of individuals during live behavioural observations. The largest female of the group (alpha female) was subordinate to the male, but dominated over the other females (beta and gamma). Each group was provided with a piece of PVC tube (7.62 cm in length and 3 cm in diameter), which the male established as a nesting site.

5.3.2 Study 1

This experiment lasted from July 5 to July 27 2011. We collected logistical data for reproductive entrainment of wild-caught fish in a laboratory environment. We constructed 12
social groups; 6 groups consisted of one male and two females and 6 groups consisted of one male and three females (male, SL = 37.28 ± 0.82 mm; alpha female, SL = 31.25 ± 0.27 mm; beta female, SL = 24.28 ± 0.63 mm).

5.3.2.1 Eggs

Once the groups were constructed, nest tubes were checked twice daily, for the appearance of eggs. Due to the combined effect of a small nest tube diameter and low lighting conditions, it is not possible to determine the stage of egg development unless the nest tube is removed from the aquarium. Thus monolayer of eggs were visually inspected by removing the tube from the aquarium and placed back immediately. We noted whether the eggs were newly laid (orange), late embryos (‘eyed’ eggs), yolk-sac larvae (transparent, with more prominent eye pigmentation) or hatched (whitish remnants of eggs without ‘eyes’). Note that we recorded the presence of clutches of eggs, but we could not reliably account for the disappearance (hatching) of individual clutches. The number of days for the first appearance of eggs and the number of days taken for the eggs to become eyed were calculated. Based on these data we also determined the percentage of clutches that became eyed. We used unpaired t-tests to determine whether there were differences in those parameters between groups consisting of two versus three females. No extensive behavioral measurements were made in this study, except that it was noted whether the male was demonstrating nest care. The limitations of this study were taken into consideration when designing Study 2.

5.3.3 Study 2

This experiment lasted for 3 weeks from July 5-31 2012. Before placing in groups, all males were weighed using an electronic balance (Sartorium). The banding pattern and size of fish were used to construct social groups (N=16), consisting of a male (SL = 42.09 ± 0.92 mm)
and two size-mismatched females (alpha female, SL = 35.29 ± 0.44 mm and beta female, SL = 30.26 ± 0.36 mm). Each nest tube was lined with an acetate sheet, which could be removed for quantification of eggs (see below). For each social group, tubes were placed in a central position within the tank, such that behavior inside the tube could be easily recorded.

5.3.3.1 Eggs

We quantified within and between group variation in egg number by counting the total number of eggs (orange + eyed) in each group at 8 different times over the 3-week period (d6, d8, d12, d16, d19, d21, d23, and d26). To quantify eggs, we lined each PVC nest tube with a custom fitted sheet of acetate. To avoid disturbing the fish, we did not check the tube for eggs before the behavioural observations. Immediately after the behavioural observations, the acetate was carefully removed from the PVC tube, placed in a frame, imaged digitally (Canon SX150 IS), and replaced into the tube. ImageJ was used to count the number of eggs. On d10, egg presence was noted, but eggs were not photographed. In long-term stable social groups with two females, there were periods of time when there were no eggs in the nest.

5.3.3.2 Behavior

Behavior observations were conducted on d6, d8, d10, d12, d16, d19, d21, d23, and d26 after initial grouping. On those days, observations began at 9 am and a live observer recorded behavior for 10 min on each group. When one individual swam within two body lengths of another individual, we recorded that behavior as an approach. When an approached fish swam away, we recorded that behavior as a displacement. In the current study, we did not evaluate whether any given behavior served an agonistic or reproductive function.

We also recorded male-specific behaviors including courtship jerks that were directed towards females, non-directed courtship jerks, and the number and duration of nest care bouts.
Nest care included vigorous rubbing and fanning inside the nest using fins (Pradhan et al., 2014). Each bout of nest care lasted from one to several seconds and each bout was separated by at least 2 s. Based on these observations, basic parameters of nest care behavior were calculated, such as average number of nest care bouts, average nest care bout duration, and average time spent fanning and rubbing eggs during the 10 min period. Rate of nest care/parenting was calculated as the number of fanning and rubbing bouts/total time spent fanning and rubbing (s). We observed that the male was always in the nest tube before it was removed for inspection. Upon replacement of the nest tube into the tank, the male entered it almost immediately.

Female courtship solicitation behavior has not been previously described in *L. dalli*, hence we used the following parameters to characterize one form of female courtship, solicitation behavior: the female must be oriented perpendicular to the male so as to display the state of her distended abdomen ( gravidity) to the male, some part of the female body must be intersected by the median linear axis of the male, the female must remain stationary and not engaged in interaction with another individual or feeding, and the female can be positioned anterior or posterior to the male (Figure 5.1). Females assumed a solicitation position for two to several seconds, but we did not record the time spent per solicitation in this study. Each solicitation bout was separated by at least 2 s. Rates of all behaviors other than parenting were calculated by dividing the number of occurrences of each behavior type by the total observation time (10 min).

In order to better understand the relationships among different behaviors and how two behaviors were connected, we constructed a transition matrix of behavioral events that occurred within 5 s of the preceding behavior (see *Statistics*). This analysis allowed us to determine the common responses of the receivers of a specific behavioral display (Okanoya, 2004). As males
can spend >90% of their time inside the nest tube (Pradhan et al., 2014), it was vital to observe behavior inside the tube. In a harem, the male and his nest is necessarily the centre of reproductive activity and behavior, therefore, we chose to observe the most important behaviors associated with the nest as a way to narrow in on those behavioral interactions critical to reproductive success. These behaviors included interactions between females, where one female approached and displaced a second female that was in the process of soliciting (i.e. associated with the nest/male). This constituted an interruption of solicitation.

5.3.3.3 Statistical analyses

A repeated measures ANOVA was used to analyze the total number of eggs produced by 16 groups on 8 different days. Our primary goal with this analysis was to determine if there was a significant among group variation in egg production over the 3 week period. Due to the large number of possible among group comparisons, we did not do further post hoc tests. A repeated measures ANOVA was also used to analyze the number of nest care bouts exhibited by 16 groups on 9 different days. Again, our primary goals were to determine if there were significant among group variations in nest care and due to the large number of possible among group comparisons, we did not do further post hoc tests. To determine whether the size of fish have an impact on number of eggs, we used simple regression analyses to analyze the relationship between the number of eggs and 1) SL of males and 2) total SL of both females. To investigate the impact of egg presence on behavior, we divided the behavior data set of all observations of each group into two categories: no eggs and eggs present. Using paired t-tests, we determined whether there was a difference in the number of days nests were without versus with eggs. To analyze whether the presence of eggs affected behavior, we performed unpaired t-tests to compare 1) the rates of nest care and 2) male approaches towards females with and without eggs.
present in the nest. We conducted a Two-way ANOVA to investigate whether three levels of status (male, alpha, beta) and two levels of egg presence (no eggs and eggs) affected the rates of courtship behavior (male jerks and female solicitation). Simple regression analyses were used to determine the relationship between the total number of eggs and 1) rate of fanning and rubbing, 2) rate of males approaching females, 3) rates of male jerks, and 4) rates of female solicitation behavior. We also used simple regression analyses to determine the relationship between the number of eyed eggs and the variables mentioned above, and between rates of alpha solicitation and rates of beta solicitation. To calculate the transition event probabilities, behavior transcriptions were converted into first order Markov chains in which rows corresponded to the preceding events and columns corresponded to the succeeding events (Klein & de Araujo), 2010. Overall totals of all the 16 groups over 9 d of observations were calculated. Ratios between number of times each transition occurred and the total number of occurrences that followed a particular event were calculated. The ratios were converted to percentage for clarity in the resultant flow chart.

5.4 Results

5.4.1 Study 1

There were no significant differences in the dynamics of egg appearance and disappearance between groups consisting of two versus three females (p > 0.05); therefore we have collapsed those groups. After groups of three fish were constructed, it took 5.75 ± 0.71 d for the first appearance of eggs. After a clutch was laid, eggs became eyed 2.42 ± 0.62 d later. Groups had 3.3 ± 0.3 clutches of eggs in 3 weeks (n=12). Most of the clutches of eggs became eyed (77.78 ± 10.12%). In groups consisting of 3 females, there were always eggs in the nest
tube; however, in groups consisting of 2 females, there were periods of time without eggs in the nest.

5.4.2 Study 2

5.4.2.1 Natural variation in number of eggs

A repeated measures ANOVA revealed a significant variation in the number of eggs produced by groups ($F_{2, 15} = 5.75, p < 0.0001$). Each group produced a variable number of eggs over the 3-week period (Figure 5.2A). For example, there was one group (Group 15) that always had eggs in the nest and another group never had eggs (Group 1). Overall, there were no differences between the total numbers of days that there were no eggs in the nest ($2.94 \pm 0.43$) versus eggs present ($3.50 \pm 0.47$) in the nest (paired t-test, $t_{15} = 0.90, p = 0.39$).

5.4.2.2 Number of eggs was not associated with size of fish

There was no relationship between total number of eggs and SL of males ($r^2 = 0.03, p = 0.54$) or total SL of females ($r^2 = 0.03, p = 0.54$).

5.4.2.3 Natural variation in nest care behavior

A repeated measures ANOVA revealed a significant variation in number of nest care (fanning and rubbing) bouts over the 3-week period ($F_{2, 15} = 5.747, p < 0.0001$, Figure 5.2B). A male from one group (Group 1) did not demonstrate any nest care behaviour. For the rest of the groups, per 10 min observation period, the average number of nest care bouts was $7.57 \pm 0.53$, the average duration of each nest care bout was $25.40 \pm 3.6$ s and the average total nest care duration was $24.82 \pm 7.0$ s.
5.4.2.4 Effect of egg presence and status on rates of behavior

There was no effect of egg presence on the rate of males approaching females (Figure 5.3A, t\textsubscript{29} = 0.47, p=0.64). Interestingly, males exhibited similar rates of nest care regardless of egg presence (Figure 5.3B, t\textsubscript{29} = 1.17, p= 0.25). A two-way ANOVA revealed a main effect of social status on courtship behaviour (F\textsubscript{2, 84} = 3.66, p= 0.03; Figure 5.4). However, egg presence did not affect rates of courtship behaviour (F\textsubscript{1, 84} = 0.26, p= 0.61). There was also no interaction between social status and egg presence (F\textsubscript{2, 84} = 0.85, p= 0.43). Bonferroni post-hoc tests revealed that when there are no eggs in the nest, beta solicitation rates are higher than directed and non-directed male jerk rates (t= 2.39, p< 0.05). Overall, 55.86 ± 0.02 % of male jerks were not directed to any particular female.

5.4.2.5 Effect of egg number on behavior

The number of eggs in the nest was not associated with male parenting, male approaches, or jerks towards females (p> 0.05; Figure 5.5). However, the total number of eggs (orange + eyed) was positively associated with rate of female solicitation (r\textsuperscript{2} = 0.25, p< 0.04; Figure 5.6A). More specifically, number of eyed eggs was strongly associated with rates of alpha solicitation (r\textsuperscript{2} = 0.39, p= 0.009; Figure 5.6B). Alpha females and beta females solicited males at similar rates (t\textsubscript{252} = 1.76, p= 0.08; See Figure 5.4). There was a positive correlation between the rates of alpha female and beta female solicitation (r\textsuperscript{2} = 0.43, p< 0.0001). A complete list of the results of these analyses is provided in Tables 5.1 and 5.2.

5.4.2.6 Description of transitions following female solicitation

A total of 875 behavioural transitions were used to generate a transition schematic (Figure 5.7). The Markov chain analyses of the behavioural transitions demonstrate that following a beta female soliciting a male, there were two main outcomes: either the alpha female
interfered with the beta female solicitation (39.2%) or the male approached the beta (45.5%). Following these alpha female interferences: 1) the beta female went back to solicit the male (34.8%), 2) the alpha female solicited the male (39.1%), or 3) the beta female approached the male (13%). In contrast, the beta female never interrupted during an alpha female solicitation of the male. Alpha female solicitation led to the males approaching the alpha female at a high frequency (77.2%). Males displaced either female a large proportion of the times that they approached them: alpha female, 99%; beta female, 100%. Displacements of each female led to either the male approaching the same female again, or the male approaching the other female.

5.5 Discussion

In a species where the sex ratio is skewed towards females and males demonstrate requisite parenting, we demonstrated that harems consisting of one male and two size-mismatched females vary substantially in their rates of egg production, both within groups over time and between groups. In long-term stable social groups of L. dalli in a semi-natural laboratory environment, we found that egg presence had no impact on rates of male or female courtship behavior. In addition, in the presence of eggs, males did not sacrifice other aspects of social behavior in exchange for nest care. Thus, there appears to be no tradeoff between male nest care or egg care and other male activities, including interaction with females, and can be considered to be able to ‘multi-task’. We show that female postural displays of gravidity (courtship solicitation) are associated with number of eggs laid. Additionally, while alpha females do not solicit more than beta females, rates of alpha solicitation are a better indicator of male reproductive success. In our experiment, there was one male and two females, and our data show that both females simultaneously sought access to the male. Transition matrix analyses
revealed intra-female competition, such that alpha females were likely to interrupt beta female solicitation, and then courted males. Taken together, these results support the hypothesis that *L. dalli* exhibit sex role reversal wherein dominant females have a significant impact on male reproductive success.

5.5.1 Egg presence had no effect on rates of courtship behavior

Many different advertisement strategies of mate quality might affect mate choice and reproductive success (Alcock, 2001). Even though secondary sexual characteristics are often involved, in fishes, the presence of eggs is also used for advertising male quality (Knapp and Kovach, 1991). Hence we considered the effect of egg presence on the rates of courtship behaviour displayed by all individuals in the social group. Importantly, we used natural changes in the cycle of egg appearance and disappearance in long-term social groups. Interestingly, in Study 2, we found that there was no effect of egg presence on rates of courtship behaviour; however, in the absence of eggs, beta solicitation rate was 3 fold higher than male jerk rate (Figure 4). When alpha and beta female solicitations are combined, females exhibit higher rates of courtship compared to males. The rates of male courtship jerks directed towards females were generally low and comprised less than half of the total jerk rates. It is possible that males court immediately before spawning, and we missed the display of male courtship during the observation sessions. Hence, in future studies, we can use continual video recordings to examine the onset of male jerks shortly before spawning. Courtship is costly and there is inter-species variation in the timing of courtship displays. For example, in damselfish, males court the day before spawning, rather than the day of spawning (Karino, 1995). In our experimental paradigm, males may also court less because the females are site attached. A consequence of our attempt to study the behaviours associated with natural variations in egg presence is that females may have
laid eggs based on the visual cues associated with egg presence. The idea is that a greater number of eyed eggs indicate that the male is good at raising young (Lindström, 1992). In other species of gobies (such as sand gobies (Forsgren et al., 1996), and desert gobies (Symons et al., 2011; Knapp and Kovach, 1991)), females tend to choose males with eggs and this may have an impact on egg numbers in our study. In future studies, number of eggs can be manipulated to determine effects on rates of male and female courtship behavior.

5.5.2 Social interactions might limit male parenting

In Study 2, nest care rate was not associated with number of eggs in the nest. Even though it is clear that the nest care bouts are normally distributed, it does not vary predictably with the total number of eggs in a nest (Figures 5.2 and 5.5). It is interesting to note that the groups where males showed highest levels of nest care were not those that had the highest number of eggs; however, when a male showed no nest care, there were no eggs in the nest.

It has been postulated that time and energy spent on courtship and territoriality may reduce the quality of parental care (time spent tending eggs), thus reducing the chances for offspring survival (Trivers, 1972). Hence parenting expenditure and life history theory predict that traits that allow a male to invest heavilily in parenting may be under a greater selective pressure compared to courtship (Trivers, 1972). In *L. dalli*, however, male courtship was not associated with eggs in the nest (Figure 5.5B). Conflict between caring for the current brood and courting females for future reproduction is also seen in the desert goby (Symons et al., 2011) and fresh water gobies (*Rhinogobidus brunneus*) (Suk and Choe, 2002). Male desert gobies provide sole parental care and females are not site attached. In the presence of females, males significantly decrease several aspects of egg care (Symons et al., 2011). The social system of *L. dalli* is slightly different, in that they live in haremic groups and majority of the females tend to
be site-attached. Due to the temporal overlap of reproductive and social behavior and the presence of overlapping broods, during the nesting phase of *L. dalli*, males perform several overlapping activities and must rapidly modulate their interaction with conspecifics. Thus time budgets and social allocation in males depend, in part, on the specific breeding system.

It is also established in mammals that parenting behavior is a form of trophallactic interaction between the parent and young, in which both parties require sensory stimulation (Rosenblatt, 2003) to exhibit behavior. We expected that nest care would not persist without the positive feedback of sensory stimulation in the form of eggs. Our results clearly demonstrate that males constitutively exhibit nest care, including nest preparation and egg care, through fanning and rubbing behaviors. Regardless of egg presence, males also approach and jerk towards females at equal rates. Thus, males do not need to reduce rates of courtship or social behavior with females in order to exhibit nest care. It has also been suggested that mating at the site of offspring care reduces the temporal conflict between mating and parenting (Stiver and Alonzo, 2009). Conflicts between parenting and mating are not likely in polygamous species with obligate paternal care because males usually have continuous breeding and multiple clutches of eggs at different stages of development (Kokko and Jennions, 2008). Based on the experimental paradigm we used here, tradeoffs may be reduced due to both these reasons. These data suggest that an optimum level of male behavior persists regardless of the context of egg presence. Exhibition of continuous nest care might be adaptive when the microenvironment around the nesting site needs to be maintained consistently. For example, flagfish, *Jordanella flordiae*, adjust their rate of nest maintenance based on whether they live in fresh versus brackish water (Hale and St Mary, 2007). Female flagfish are likely to spawn with males that tend nests before spawning has occurred, but this happens only in cases when fish live in fresh water (Hale
and St Mary, 2007). In the present we used water directly supplied from the ocean and we do not have sufficient data to assess microenvironments around the nests.

In our behavioral assay, we did not measure specific aspects of fanning behavior, fin size, or brood defense, involving the male actively chasing out females from the nest. These traits can vary with reproductive status, egg presence or developmental stage of eggs (Wantola et al., 2013; Torricelli et al., 1985). For example, the type of fin used, the body positioning (Torricelli et al., 1985) or active versus passive fanning (Zoran and Ward, 1983), can vary. Courtship vigor is proportional to mating success and male parenting quality in the bicolor damselfish, Stegastes partitus, a polygamous species in which males provide sole parental care (Knapp and Kovach, 1991). Hence it is possible that L. dalli can modify the specifics of their fanning and rubbing behavior based on egg development stages, presence of eggs, or female availability.

Several hypotheses could explain why males display nest care behaviour constitutively. First, males might be laying down sperm trails, as is the case in other goby species (Marconatto et al., 1996). Second, males might display fanning to increase aeration to keep the nesting tube clean and free of disease. In this study, as the breeding season progressed, many of the acetate sheets were coated with a thin layer of algae, and continual fanning might reduce the growth. Third, display of male nest care could induce females to breed. As mentioned earlier, in many species, females tend to lay eggs in nests where males display high rates of nest care (Forsgren, 2007; Forsgren et al., 1996; Karino and Arai, 2006); hence males may constitutively display fanning behaviours. Fourth, fanning might aid in dispersing pheromones into the water for short-range chemical signaling towards conspecifics (Sorensen et al., 2005). Overall, we found no evidence for tradeoffs in male courtship or nest care behaviors.
5.5.3 Female biased sex ratio permits intra-female competition and sex role reversal

The details of female mating behaviour in *L. dalli* have not been described previously. Considering that the operational sex-ratio is female biased and brood care is only provided by males, we hypothesized that the conventional sex roles would be reversed. We found that even though alpha females do not court males more than beta females, they have a greater impact on male reproductive success (Figures 5.4 and 5.6). There was also a positive relationship between rates of alpha solicitation and beta solicitation and alpha females frequently interrupted beta female solicitations (Figure 5.7). Social rank appears to bias interactions among females, with higher-ranking individuals approaching and displacing a soliciting subordinate, but not the converse. In stable social groups, female aggression and status can be a predictor of nest access (Solomon-Lane et al., 2014), such that alpha female agonistic behaviours are negatively associated with reproductive success. Here, we found that alpha female solicitation was positively associated with number of eyed eggs and transition matrix analyses demonstrate that alpha females frequently interrupt beta female solicitation. As a result of reduced availability of beta females for males, males respond at different frequencies to solicitation by alpha versus beta females.

Preliminary studies show that in *L. dalli*, alphas have priority in egg-laying compared to subordinate females (beta and gamma), but it is unknown whether they lay more eggs (Solomon-Lane and Grober, unpublished results). In future, it will be informative to track egg laying and hatching success of eggs from females of different statuses. In many species with diverse mating systems, dominant individuals have more access to copulate compared to subordinate individuals. For example, in a cooperatively breeding cichlid, *Neolamprologus pulcher*, dominant individuals reproduce and actively suppress the reproduction of subordinate
individuals, who help the dominant individuals with broodcare (Balshine-Earn et al., 1998; Heg, 2008). In the angelfish, *Centropyge bicolor*, a harem hermaphroditic species, similar to *L. dalli*, the higher social rank in females is associated with greater reproductive success (Ang and Manica, 2010). Further studies are required to assess the individual female contributions to the overall fitness in *L. dalli*. Finally, our analysis of egg production showed that rates of evidence female courtship are positively associated with male reproductive success and provides robust evidence for sex role reversal in *L. dalli*. Taken together, our work on *L. dalli* demonstrates that alpha females are important regulators of male reproductive success and as such, must be considered when evaluating the contribution of sexually dimorphic behavioural dynamics to fitness in this species.

5.6 Acknowledgements

We thank Cory Grober and Caitlin McCoyd for help with technical support and the staff at USC Wrigley Institute for Environmental Studies for logistical assistance. We are grateful for the grants from NSERC PGS D3, Sigma Xi, Brains & Behavior Program at Georgia State University and Georgia State University Dissertation Award to DSP, NSF (IOB – 0548567) to MSG, and NSF Doctoral Dissertation Improvement Grant (1210382) to MSG and DSP.

5.7 References


Marconatto, A., Bisazza, A., 1986. Males whose nests contain eggs are preferred by female


Swierk, L., Myers, A., Langkilde, T., 2013. Male mate preference is influenced by both female behavior and morphology. Anim. Behav. 85, 1451-1457.


Table 5.1: Relationship between total number of eggs (orange + eyed) and behavior of *L. dalli*

<table>
<thead>
<tr>
<th>Behavior</th>
<th>$r^2$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of fanning and rubbing</td>
<td>0.03</td>
<td>0.50</td>
</tr>
<tr>
<td>Rate of male approaches</td>
<td>0.20</td>
<td>0.09</td>
</tr>
<tr>
<td>Rate of male jerks</td>
<td>&lt;0.001</td>
<td>0.10</td>
</tr>
<tr>
<td>Rate of alpha + beta solicitation</td>
<td>0.25</td>
<td>0.04*</td>
</tr>
<tr>
<td>Rate of alpha solicitation</td>
<td>0.17</td>
<td>0.12</td>
</tr>
<tr>
<td>Rate of beta solicitation</td>
<td>0.03</td>
<td>0.55</td>
</tr>
</tbody>
</table>

n=16 groups, statistically significant p values are in bold; p< 0.05
Table 5.2: Relationship between number of eyed eggs and behavior of *L. dalli*

<table>
<thead>
<tr>
<th>Behavior</th>
<th>$r^2$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of fanning and rubbing</td>
<td>&lt;0.01</td>
<td>0.82</td>
</tr>
<tr>
<td>Rate of male approaches</td>
<td>0.13</td>
<td>0.17</td>
</tr>
<tr>
<td>Rate of male jerks</td>
<td>0.11</td>
<td>0.21</td>
</tr>
<tr>
<td>Rate of alpha + beta solicitation</td>
<td>0.15</td>
<td>0.14</td>
</tr>
<tr>
<td>Rate of alpha solicitation</td>
<td>0.39</td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td>Rate of beta solicitation</td>
<td>&lt;0.001</td>
<td>0.92</td>
</tr>
</tbody>
</table>

n=16 groups, statistically significant p values are in bold; p< 0.05
Figure 5.1: An illustration showing scenarios and rules for scoring courtship solicitation postures by *L. dalli* females (top views). The female must align her body perpendicular to the male so as to display the state of her distended abdomen (gravidity) to the male, the female must remain stationary and not engaged in interaction with another individual or feeding. (A) The female could be positioned anterior or (B) posterior to the male (C) Some part of the female body must be intersected by the median linear axis of the male.
Figure 5.2: groups of *L. dalli* show within and between group natural variation in (A) Average total number of eggs (orange + eyed) laid on 8 different days (B) Average number of nest care (fanning and rubbing) bouts exhibited on 9 different days during 10 min observation sessions, over the course of 3 weeks in social groups housed in a semi-natural laboratory environment. Each social group (N=16) consisted of one male and two size-mismatched females. Error bars for each group captures variation within groups and across time.
Figure 5.3: Effect of egg presence on rates of *L. dalli* male behavior (A) male approaches towards females (B) male parenting over a 3 week period. Each group comprised of one male and two status mismatched females. Eight behavioral observations were conducted for N=16 groups.
Figure 5.4: Effect of egg presence on rates of courtship behavior exhibited by *L. dalli* living in groups comprised of one male (M) and two status mismatched females (α= alpha female, β= beta female) during egg absence and presence over a 3 week period. When there are no eggs in the nest, rates of beta solicitations are higher than males jerk rate. Eight behavioral observations were conducted for N=16 groups. The letters inside the histogram represent sex-specific courtship behavior, J= male jerks, S= female solicitation, *p*<0.05.
Figure 5.5: Relationship between total number of eggs (orange + eyed) and rates of *L. dalli* male behavior (A) proportion of time spent parenting (fanning and rubbing) (B) male courtship jerks (C) male approaches towards females. Nine behavioral observations were conducted for N=16 groups over a 3 week period.
Figure 5.6: Relationship between number of eggs and rates of *L. dalli* female behavior (A) alpha + beta solicitation and total number of eggs (B) alpha solicitation and number of eyed eggs.

Nine behavioral observations were conducted for N=16 groups over a 3 week period.
Figure 5.7: Simplified flow chart showing the pattern of interactions within *L. dalli* groups consisting of one male, an alpha female, and a beta female, based on first order Markov transitions. A total of 875 transitions were used to generate the chart, and arrow thickness is proportional to the frequency (converted into percentage, and noted beside the arrow) of the transition. To reduce complexity, only frequencies >0.1 (10%) are shown.
6 TISSUE STEROID LEVELS ARE ASSOCIATED WITH FEMALE SOCIAL STATUS, BUT NOT RATES OF BEHAVIOR NOR OVARIAN FUNCTION

6.1 Abstract

A variety of factors affect circulating and/or tissue hormone levels in females during the breeding season. Cyclical changes in ovarian hormones influence context-specific interactions and can confound simple hormone-behavior relationships. Here, we examined the roles of morphology, behavior, and female social status in establishing brain and ovarian levels of steroid hormones in females of a bi-directionally hermaphrodite fish, the bluebanded goby, *Lythrypnus dalli*. The alpha female maintains the highest social status among females in a harem and is subordinate to the male. Male reproductive success is associated with agonistic and courtship behavior of alpha females. For 4 weeks, we observed social groups (n=34), consisting of one male and two status-mismatched females. As expected, there were significant morphological differences between alpha and beta females, however, there were no relationships between steroid levels and morphology nor rates of agonistic interactions. Short-term, transient changes in social context also did not affect steroid levels. Interestingly, beta females had 2x higher brain levels of testosterone, 11-ketotestosterone, and cortisol than alpha females; however, alpha females had higher ovarian 17β-estradiol levels. Lower brain androgens and glucocorticoids in alpha females might minimize physiological costs and permit transient elevation during sustained changes in social context. Brain 17β-estradiol levels were dramatically lower compared to the ovary and the ratio of steroids were different for brains and ovaries, likely due to local synthesis and regulation of steroids. Despite changes in short-term context and the high
variation in ovarian morphology, dominant and subordinate females maintain robust tissue-specific differences in steroid hormones.

### 6.2 Introduction

Species living in complex social groups display a repertoire of context specific behaviors steroid hormones may regulate these behaviors (Angelier et al., 2007; Oliveira, 2009; Ostner et al., 2008; Renn et al., 2012). Apart from year-round seasonality (Prendergast et al., 2009), during the breeding season, circulating steroid hormones can vary based on specific mating systems (Creel, 2001), nutrition (Schneider and Wade, 1999), degree of parental care (Wingfield et al., 1990; Wingfield et al., 1999), sex (Renn et al., 2012), social status (Cardwell et al., 1996; Fox et al., 1997; Terburg and van Honk, 2013), age (Angelier et al., 2007; Beehner et al., 2006), gonadal development (Prendergast et al., 2009) and reproductive state or phase (Wingfield et al., 1990). In addition, steroid hormone levels also temporarily fluctuate during critical periods of social instability (challenge hypothesis) and are counterbalanced with lower ‘breeding-baseline’ levels during stable periods (Beehner et al., 2006; Wingfield et al., 1990). All of these variables often co-occur and are difficult control, especially when studying wild populations. To investigate the mechanisms underlying social behavior, studies are often conducted in captive populations within a limited social environment.

Most studies that examine the relationship between social behavior and steroids in wild or semi-wild conditions have focused on males and less is known about how steroids influence social behavior in females. One reason for this is because in some systems, like songbirds, males are easier to observe and trap. Second, ovarian cyclicity can complicate the simplistic hormone-behavior relationship due to fluctuations in steroidal milieu (Becker et al., 2005; Mogil and
Chanda, 2005). Oogenesis in all female vertebrates requires a careful orchestration of various steroid hormones to achieve final oocyte maturation (Nelson, 2011). In Teleost fishes, gross morphological measurements are often used to track changes in ovarian function. Two measures of gonadal function, gonadosomatic index (GSI) and ovarian score may help discern the relative importance of traditional and non-traditional hormone sources. Variations in GSI and ovarian morphology occur due to endocrine regulation of ovulation through the reproductive season as females cycle. Gonadosomatic index, the ratio of gonad weight to body weight, is a widely used conventional method of determining gonadal development to forecast spawning seasons when females have reached high gonadal maturity (Bandpei et al., 2011; Kokokiris et al., 2000). Gonadal maturity can also be categorized using ovarian score, which uses varying degrees of gross morphological traits as oocytes become hydrated (Skoblina, 2009). It is important to note that ovarian score is a subjective measure and can vary from experiment to experiment. However, larger female fish consistently have larger ovaries than their smaller counterparts (Drilling and Grober, 2005; Sisneros et al., 2004). Variation in GSI and or ovarian score may be associated with steroid hormones and several studies report annual or seasonal cycles of plasma hormone levels with these morphological traits (Sisneros et al., 2004), but the levels of steroids within the ovary are seldom reported.

Bluebanded gobies, *Lythrypnus dalli* are an excellent system to determine the relative importance of different factors in influencing tissue levels of steroid hormones. These fish are highly social, harem, conventional sexual role reversed, and capable of adult sexual plasticity caused by disruption of a stable hierarchy. Wild-caught fish can be housed in small social groups in a semi-natural laboratory environment. While the male is the most dominant individual in a group, females form a linear social hierarchy. Male reproductive success is
associated with agonistic (Solomon-Lane et al., 2014) and courtship behavior (Pradhan D. and Grober M. unpublished results) of alpha females. There are no sex (Lorenzi et al., 2008) or female status (Solomon-Lane T. and Grober M., unpublished results) differences in levels of water-borne steroid hormones. Tissue steroid hormone levels however, differ among status classes in stable groups of *L. dalli* (Lorenzi et al., 2012). Tissue steroid levels are affected by differences in social environment (Black et al., 2005; Lorenzi et al., 2012; Pradhan et al., 2014a; Pradhan et al., 2014b). While the sex change process induces rapid, transient, and dynamic changes in tissue steroid hormone levels in females of all statuses, little is known about hormone levels in stable social groups (Lorenzi et al., 2012). In the previous study involving *L. dalli* the status differences in female tissue steroid hormones were from social groups that had been stable for one week, but factors underlying those differences were not evaluated (Lorenzi et al., 2012). No attempt was made to observe female ovarian cycles and evaluate mechanisms responsible for maintaining differences in status in stable groups or how transient changes in social context affect steroid levels in stable groups.

Here, examined four types of steroid hormones that may influence ovarian maturation and female social status: 11-ketotestosterone (KT, a potent teleost androgen) and testosterone (T), 17β-estradiol (E₂), and cortisol (F, the potent teleost glucocorticoid). These steroids are linked through steroidogenic conversion pathways, such that T can be converted to E₂ by the enzyme aromatase and it can also be converted to KT by a two-step process. The enzyme 11β-hydroxylase converts T to 11-hydroxytestosterone, which is then converted to KT by 11β-hydroxysteroid dehydrogenase (11β-HSD). Finally, F can be deactivated to cortisone by 11β-HSD. We evaluated both brain and ovarian steroids because both of these hormone sources may be important for regulating morphology and/ or behavior. Thus the internal steroidal milieu and
consequent phenotypic effects could be maintained or changed through the increase or decrease in one or more of these steroids. For example, brain and gonadal KT is transiently up-regulated while E₂ decreases during protogynous sex change (Lorenzi et al., 2012). This process is also accompanied by a decrease in aromatase activity (Black et al., 2005). 11-Ketotestosterone is also associated with male parenting behavior and inhibition of 11β-HSD activity reduces the ratio of KT to F (KT:F) (Pradhan et al., 2014b; Rodgers et al., 2006). However, it is not clearly understood whether KT affects ovarian maturation (Kokokiris et al., 2000). In other species, it has been hypothesized that ovarian development can be directed in part by regulating levels of T (Frisch, 2004; Sisneros et al., 2004). Ovulation is marked by an initial increase in T and E₂, followed by a drop in plasma levels once oocytes have matured completely (Kokokiris et al., 2000; Peter and Yu, 1997). Previous research also shows that E₂ facilitates sexual transition, directs gonadal development, and establishes social status (Frisch, 2004). Cortisol has also been implicated in regulating reproductive function, for example, stress-induced secretion of F suggests social status can dictate the rate of ovulation as a reproductive tactic in dominant fish (Gennotte et al., 2012). Yet, it can be found at large levels in developing oocytes where its origins and role are still ambiguous (Brooks et al., 1997). Region-specific bioavailability and subsequent interplay between these hormones may provide insight into the synchronization of physiology and behavior during the reproductive season.

Taken together, during a complicated social situation, many different factors can affect hormone levels in group-living animals. Behavioral changes in one individual can lead to both, behavioral and physiological changes in conspecifics (Cheng, 1983; Hutchison and Steimer, 1985). In addition, pre-existing ovarian morphology and social status might also prime the individual for expressing behavior and physiological changes in response to social instability.
Here, we first examined the morphological and hormonal differences between alpha and beta females to determine the physiological mechanisms may be responsible for maintaining a difference in status. As a result of another study with males (Pradhan et al., 2014b), we investigated effects of transient changes in social context on stable groups of fish. In both these cases, we evaluated whether pre-established social status (dominant and subordinate) underlies differences in levels of four different steroid hormones (T, E$_2$, KT, and F), collected from two types of tissues (brain and ovary).

6.3 Methods

6.3.1 Subjects

All fish for these experiments were collected off the coast of Catalina Island, California July of 2012 by SCUBA diving and using hand nets (permit number SC-11879). After capture, the fish were kept in 2 L plastic Nalgene bottles, brought to the boat, and then placed in a large bucket for transport to a laboratory at the Wrigley Institute for Environmental Studies. Fish were housed in 60 x 94 cm$^2$ aquaria with continuous seawater and exposed to natural ambient light cycles. After anesthetization with tricaine methanosulphate (MS-222; 0.5 mg/100 mL water) measurements were taken under a dissecting microscope, including standard length (SL), verification of sexually dimorphic genitalia, and banding pattern (for identification of individuals). A total of 34 social groups, each consisting of one male (SL= 41.44 ± 0.53 mm) and two size-mismatched females (SL: alpha= 35.13 ± 0.29 mm; beta= 30.72 ± 0.24 mm) were constructed in smaller 31 x 20 cm aquaria. These differences in size usually assure a linear social hierarchy in the group (Reavis and Grober, 1999a). Each social group was provided with
a piece of PVC nest tube, housed for a period of 4 weeks and had at least two clutches of eggs during this time. Animals were fed twice daily, at 8:00 and 16:00 h with frozen brine shrimp.

6.3.2 General procedures

On the day of sampling, baseline behavioral observations were conducted to verify the dominance hierarchies of the social groups. Within seconds after the behavior observations, male manipulations for a different experiment were conducted (Pradhan et al. 2014b), while alpha and beta females were placed in separate cups for $27.55 \pm 2.05$ min to ensure that they did not have access to the unprotected eggs and that dominant females did not initiate behavioral and physiological transformations associated with sex change in the absence of the male. Placement of isolated fish in cups for up to 1 h away from a group is a routine procedure in our laboratory and does not disrupt established social hierarchies among familiar individuals (Pradhan et al., 2014a). When animals were returned to the group, they were allowed to interact for 60 min. During this time, behavioral observations were conducted (see below) and immediately after, animals were euthanized with excess MS-222 (1 mg/100 mL water placed on ice).

6.3.3 Morphological measurements

Upon euthanasia, each fish was dabbed with Kimwipe to absorb excess moisture, placed on a scale (Sartorium) to record the body weight, and then placed under a dissecting microscope to measure SL. Next, the brain was rapidly removed and frozen on dry ice, followed by the ovaries. Upon removal of the ovary, the ovarian score was recorded according to the following scale: 1= early in cycle, 2= mid-cycle, 3= late in cycle, and 4= end of cycle (Figure 6.1). Time taken from euthanasia to tissue removal did not differ between females of the two status classes (average time: alphas= $5.46 \pm 0.16$ min; betas= $5.41 \pm 0.20$ min; $t_{66}= 0.17$, $p= 0.86$). Samples were then stored at $-80^\circ C$ for 7 d, packed on dry ice, sent to Georgia State University on dry ice,
and then stored at -80°C for 7 months until assays. On the day of tissue processing, each tissue was placed on aluminum foil on dry ice, split into two and then weighed (Meter Toledo). Half the tissue was then placed in ice-cold phosphate buffer (0.1 M, pH 7.8) and homogenized for hormone extractions (see below). The GSI was calculated according to the following formula: 

\[(\text{Ovary weight} / \text{body weight}) \times 100.\]

### 6.3.4 Behavior measures and social contexts

Before male manipulation, baseline behavioral observations were conducted for a period of 10 min. After male manipulation, males were introduced into the home tank and it was noted whether the male entered the nest within 60 s. Sustained nest territoriality is generally exhibited in the most dominant individual of the social group (Pradhan et al., 2014a; Pradhan et al., 2014b). Following 60 s, females were introduced into their respective home tanks and the behavioral interactions among individuals in the group were noted for a period of 60 min. For males that had not previously entered the nest, time of first nest entry was noted. We compared hormone levels in alpha and beta females from groups where males had entered the nest before the females were introduced to groups (n=12) with those where males exhibited delayed nest entry (n=22). The former set of groups reached social stability rapidly and the latter set of groups varied in the time taken to reach stability. Social interactions among group members, time spent by each individual in the nest, and parenting behavior inside the nest were quantified in 10 min intervals for the first 30 min, and then from 50 to 60 min. In cases where females entered the nest, some alpha females demonstrated male-typical parenting behavior (Pradhan et al., 2014b). For the second social context, we compared hormone levels in females from groups where alpha females exhibited parenting to those in which the alpha female did not exhibit parenting (n=22).
We quantified agonistic behaviors using previous sampling procedures (Pradhan et al., 2014a), which comprised of approaches (when one fish came within two body lengths of another fish) and displacements (if the approached fish responded by swimming away). Based on these observations, we calculated a composite score of aggression, “agonistic efficiency” (Pradhan et al., 2014a; Solomon-Lane et al., 2014). It measures the success rate of an individual’s approaches by dividing the rate of displacements by the rate of approaches. Fish of a higher status generally have a high agonistic efficiency. In cases when an individual did not approach any other conspecific, agonistic efficiency was incalculable. Rates of behavior were calculated by dividing the number of occurrences of each type of behavior by 10 min, which was the length of each observation period. We determined agonistic efficiency of each individual for baseline observations and for the last 10 min of the behavioral observation, prior to euthanizing the animals.

6.3.5 Steroid hormone assays

All procedures for tissue preparation, homogenization, C18 extractions, and enzymeimmunoassays (EIAs) were performed according to previously published methods (Pradhan et al., 2014a), with some modifications. Briefly, all tissues were homogenized in 500 µL ice-cold phosphate buffer, shaken for 1 h, and stored overnight at 4°C. The following day, solid phase extractions were conducted using C18 columns (Sepak 6 mm, 500 mg sorbent) fitted to a 24-port manifold attached to a vacuum pump. Sample eluates were dried with nitrogen and re-suspended to yield a final volume of 500 µL (7.5% ethanol and 95% EIA buffer supplied by Cayman Chemical kits), parafilmed individually, and stored at -20°C. Prior to steroid hormone measurements, samples were shaken on a multi-tube vortex for 1 h and all the directions provided in the kit insert were followed. These procedures have been previously validated for L.
*dalli* (Lorenzi et al., 2012; Pradhan et al., 2014a). All samples were assayed in duplicate. Details on the cross-reactivity of the assay and quality of standard curves can be found on the supplier’s (Cayman) website (https://www.caymanchem.com/app/template/Product.vm/catalog/582751). Following the addition of Ellman’s Reagent, plates were read using a Victor X Multilabel Plate Reader (Perkin Elmer). As per the recommendations of the manufacturer, absorbance wavelength was set between 410 and 415 nm. All data are presented as pg /mg tissue (pg /mL multiplied by the volume the dried sample was initially suspended in and adjusted for the dilution if necessary and further corrected by tissue weight. The intra-assay variations were 6.4% for T, 5.7% for KT, 10.5% for E$_2$ and 3.4% for F, while the inter assay variations were 6.7% for T, 9.4% for KT, 11.7% for E$_2$ and 3.8% for F. For ovarian E$_2$ some samples had to be diluted 10x to get a reading within the reliable range of the standard curve, but this was not sufficient for most samples. Hence, in those cases, we set the numbers to the highest detected amount for calculation of average ovarian E$_2$.

### 6.3.6 Statistical analyses

All data shown here are mean ± S.E.M. and were analyzed using Prism 4.0 for Mac. Data were transformed to achieve homogeneity of variance and normal distribution or non-parametric alternatives were used where appropriate. Morphological differences such as SL, body weight, brain weight, ovarian score, and GSI between alpha females and beta females were evaluated using unpaired t-tests. Linear regression analyses were used to examine the relationship between 1) SL and each steroid hormone, 2) body weight and each steroid hormone, 3) GSI and ovarian score for all females 4) ovarian score and each steroid hormone, and 5) between GSI and each steroid hormone. We determined whether ovarian score categories had an impact on ovarian steroid hormone levels by one-way ANOVAs, followed by post hoc Tukey’s
Multiple Comparison tests. In order to assess whether disturbance of the social group after the 4-week period had effects on behavior of individuals, we performed sets of two-way ANOVAs followed by post hoc Bonferroni t-tests. First, we analyzed whether time (baseline versus post male treatment) had an effect on rates of agonistic efficiency of individuals of each status class (male, alpha female and beta female). Second, we analyzed whether time (baseline versus post male treatment) had an impact on duration in the nest by individuals of each status class (male, alpha female and beta female). To determine whether social context had an effect on hormones, we conducted two-way ANOVAs for each type of hormone. In the first case, we evaluated the effect of male behavior (in nest or not in nest) and female status (alpha: dominant and beta: subordinate) for each type of hormone. Next, we evaluated the effect of alpha female parenting (in nest or not in nest) and female status (alpha: dominant and beta: subordinate) for each type of hormone. Significant main effects were evaluated with t-tests. Finally, used a subset of females to calculate the ratio of two hormones (KT:T, KT:E2, KT:F, T:E2, T:F, and F:E2) and compared these ratios between alpha females and beta females. Given that steroidogenic enzymes convert hormones from one form to another and the same enzyme can participate in multiple pathways, the relative amounts of the steroids, rather than the absolute levels might be important to consider. Only those females whose ovarian E2 levels were within the detectable range of the standard curve were used for these analyses. All tests were two-tailed and alpha was set at 0.05.

6.4 Results

6.4.1 Morphology

At the time of group construction, females were assigned to groups based on their sizes and these differences were also maintained at the end of the experiment (Table 6.1). All the
other morphological assessments were performed at the end of the experiment and confirmed that there were multiple differences between dominant and subordinate females. Interestingly, while ovarian scores of alpha females were significantly greater than beta females, their GSIs were not significantly different (Table 6.1). However, beta females had a greater range of GSI compared to alpha females (Figure 6.2, alpha females GSI range: 1.66–12.7; beta females GSI range: 0.57–20.84). More than half the beta females (56%), but only 21% alpha females had a stage 2 ovarian score, while 47% alphas females and < 1% beta females had a stage 4 ovarian score. For all females, ovarian score was positively associated with GSI ($r^2 = 9.4676$, $p < 0.0001$). Ovarian score, GSI, SL, and weight of fish were not associated with any steroid hormone from brain or ovary ($p > 0.05$ in all cases). When comparing categorical effects of ovarian score on E$_2$ through one-way ANOVA, we found significant effects ($F_3 = 5.434$, $p = 0.002$; Figure 6.3), but not any other hormone. Levels of E$_2$ were lowest for stage 1 compared to all other stages (1 versus 2: $q = 4.07$, $p < 0.05$; 1 versus 3: $q = 5.54$, $p < 0.01$; 1 versus 4: $q = 4.77$, $p < 0.01$).

6.4.2 Behavior

To confirm the status of each individual in the social group, we compared the agonistic efficiency of individuals before and after male treatment (Figure 6.4). There was a main effect of female status ($F_2 = 66.40$, $p < 0.0001$), but no main effect of time (baseline versus after male treatment: $F_1 = 0.002$, $p = 0.96$), and no status x time interaction ($F_{1,2} = 0.67$, $p = 0.52$). Males had the highest agonistic efficiency (male versus alpha female: baseline, $t = 3.90$, $p < 0.001$ and post male treatment, $t = 4.25$, $p < 0.001$; male versus beta female: baseline, $t = 8.69$, $p < 0.001$ and post male treatment, $t = 7.47$, $p < 0.001$), while beta female had the lowest (alpha female versus beta female: baseline, $t = 5.50$, $p < 0.001$ and post male treatment, $t = 4.03$, $p < 0.001$). There were no relationships between the rates of behavior or agonistic efficiency and levels of steroid hormones.
(p> 0.05). There was a main effect of status on duration in nest (F_{2} = 220.8, p< 0.0001), a main effect of time on duration in nest (F_{1} = 0.30, p= 0.58), and duration in nest x time interaction F_{1}, 2= 24.82, p< 0.0001; Figure 6.4B). Even though the differences in duration in nest were maintained among the individuals, males spent lower amount of time in nest after treatment, while alpha and beta females were in the nest longer after male treatment (p< 0.05).

For the first social context, we investigated whether male behavior had an effect on levels of steroid hormones in alpha and beta females (Table 6.2). There were no significant main effects of male nest presence on brain or ovarian steroid hormones (p >0.05) in alpha or beta females. However, there were significant main effects of status on levels of T, KT, and F in brain, but not E_{2}. Interestingly, the effects on ovarian steroid hormones were reversed, such that there were main effects of status on E_{2}, but not any other steroid hormone (Table 6.2). There were also no significant interactions between male nest presence and female status for any steroid hormone in brain or ovary. Next, we examined whether alpha female parenting behavior had an effect on levels of steroid hormones in alpha and beta females. While there were no main effects of status or alpha parenting on ovarian steroid hormones, there were significant main effects of status on brain androgens and F, but not E_{2}. There were no significant interactions between alpha parenting and female status for any steroid hormone in brain or ovary (p> 0.05).

To breakdown the significant main effects of status, the behavioral categories were collapsed and differences between alpha females and beta females were analyzed (Figures 6.5, 6.6). Levels of both androgens and F were higher in the brains of beta females (T, t_{66}= 5.17, p< 0.0001; KT, t_{66}= 3.91, p< 0.0002; F, t_{66}= 2.98, p< 0.004), while ovarian E_{2} was higher in alpha females (t_{66}= 2.09, p= 0.04).
6.4.3 Ratios

The relative concentrations of KT:E₂, T:E₂, and F:E₂ were all higher in brains of beta females compared to alpha females (p< 0.05; Figure 6.7A, Table 6.4). Additionally, T:E₂ and T:F were also higher in ovaries of beta females compared to alpha females (p< 0.05; Figure 6.7B, Table 6.4).

6.5 Discussion

We demonstrate that female status had a tremendous impact on tissue hormone levels, such that subordinate females had higher brain levels of both androgens and the glucocorticoid, and dominant females had higher ovarian levels of an estrogen. In addition, subordinate females also had a lower body weight, brain weight, ovarian weight and ovarian score compared to more dominant females, but none of these phenotypes were associated with tissue steroid levels. Interestingly, despite the dramatic variation in E₂ levels, there were no associations with GSI and ovarian score; however, ovarian score could be used to predict whether a female might have high or low E₂ levels. Short-term changes in social context did not affect the established status and steroid hormone levels. The observations that brain E₂ levels were dramatically lower compared to the ovary and that the ratio of steroids were different for brains and ovaries, support the hypothesis that the brain is able to locally regulate E₂ levels and is not a passive recipient of E₂ from the ovary. Using tissues as a proxy for steroid levels may be more informative and help explain discrepancies from systemic data. Despite changes in short-term context and the high variation in gonadal morphologies, dominant and subordinate females maintain robust tissue-specific differences in steroids. It is important to note that in this experiment, all animals in the social group were familiar with each other, interacted on a long-term basis, and maintained
status-specific agonistic efficiency (Figure 6.4A). This is different from most other studies that only measure steroids after brief simulated encounters. Reciprocal behaviors can influence steroid levels in both individuals, but there are also sex differences in response to social encounters. For example, after a contest, in a cichlid fish, female winners do not show elevated plasma T or KT, but males do (Taves et al., 2009). However, again, tissue-specific steroid responses might be different, but it was not investigated in that study.

### 6.5.1 Morphology does not influence status specific steroid hormone levels

In this experiment, social groups were constructed based on differences in SL of females. While the differences in SL were maintained at the end of the experiment, other biometric measures were also found to be different. Body, brain, and ovarian weights were all higher in alpha females (Table 6.1). Interestingly, while ovarian scores were higher in alphas females compared to beta females, GSI did not differ, similar to a previous study on female cichlids (Renn et al., 2012). This suggests that even though the beta female is smaller, her ovarian weight compensates proportionally, while alpha females have bigger bodies and larger ovaries, but not disproportionately. These data indicate that beta females are reproductively active. Even though the ovary has a cyclical nature, very few females had ovaries in stage 1, and they were all beta females. On the other hand, majority of alpha females tended to have a higher ovarian score. This could be explained by at least two hypotheses. First, it is possible that beta females had recently laid eggs and as a result their ovaries appeared spent. Second, it is possible that beta females generally tend to produce and lay fewer eggs compared to alpha females. There are many examples where intrasexual competition leads to the more dominant individuals in a social group suppressing the reproductive maturation in subordinates (Ang and Manica, 2010; Heg, 2008). In *L. dalli*, using a similar semi-natural group set up such as the present experiment, but
consisting of three females, the most subordinate female had the lowest gravidity score (a morphometric measure of distended belly containing eggs) over a period of 5 weeks (Solomon-Lane T., Pradhan D. and Grober M., unpublished results). In the present experiment, the beta was the lowest ranking individual and might be cycling similar to a gamma female in groups consisting of three females. Due to the skewed representation of the ovarian score in the sample the categorical effect of ovarian scores on steroid hormone levels must be interpreted with caution. However, it is interesting that $E_2$ was the only hormone on which the ovarian score had an impact, and the levels of this hormone were generally extremely high in the ovary.

### 6.5.2 Regardless of context, females maintain status specific steroid hormone levels

Short-term changes in social context did not affect tissue hormone levels in females (Tables 6.2 and 6.3). With regards to male nest presence, males from 12 groups were already in the nest before females were re-united with the social group. Among the remaining groups, the latency for male nest entry was highly variable and created a unique situation that permitted females to enter the nest in the absence of the male. While both alpha and beta females entered the nest, only the alpha female demonstrated egg care, indicating the initiation of male-typical behaviors. If alpha female territoriality had persisted for a longer period of time, the alpha female would change sex completely (Reavis and Grober, 1999b). Interestingly, in the alpha females that demonstrated parenting, brain KT was elevated compared to those alpha females that did not parent (Pradhan et al., 2014b). Brain KT promotes parenting in male $L. dalli$ (Pradhan et al., 2014b) and KT also elevates in the brain of alpha females undergoing sex change within 24 h (Lorenzi et al., 2012). The effect of alpha female parenting on brain KT was not significant in the present study, even though it was the same data set, probably because of the two-way ANOVA here, compared to a t-test reported earlier. In addition, the elevated brain KT
levels in the alpha females of the present experiment had not reached the level of the beta females. This could be because all the contextual changes were of a very short duration and lasted for varying amounts of time. As a result, there are many limitations of this analysis. However, it cannot be ignored that complex social hierarchies present many challenges and even if we account for many variables in a well-designed experiment, the qualitative nature of interactions among conspecifics cannot be controlled for, and are seldom consistent across experimental groups. Regardless of a complicated social situation in this study, tissue steroid hormone differences persisted in a similar pattern and this indicates that these status-specific hormones are robust. This study lays the foundation to investigate neuroendocrine mechanisms that allow for the maintenance of tissue and status specific hormones in naturally cycling females.

6.5.3 **Tissue hormones indicate status**

In this study, subordinate females had higher levels of androgens compared to dominant females, as found in the previous study (Lorenzi et al., 2012). This finding is contrary to what is generally seen in other species such as trout (Cardwell et al., 1996) and cichlids (Renn et al., 2012). However, this may be because we report tissue androgen levels, whereas past studies report plasma hormone levels. There are also no status differences in steroids exuded in water, a measure of systemic hormones, in female *L. dalli* (Solomon-Lane, Pradhan, and Grober, unpublished results). In female rock hyraxes, subordinate individuals have elevated androgens in the fur, and fur quality is a direct measure of fecundity (Koren and Geffen, 2008; Koren et al., 2006). Taken together, these data indicate that the proxy of steroid measure must be considered carefully when designing experiments, drawing conclusions, and comparing results across experiments. By measuring tissue hormones, we have measured hormone levels that are
potentially bioactive and are very close to the site of cellular steroid actions (Pradhan et al., 2014a; Schmidt et al., 2008). There are many pieces of evidence that support the idea that the brain can independently regulate steroid levels and behavior without cues from the periphery (London et al., 2009; Lorenzi et al., 2012; Pradhan et al., 2010; Pradhan et al., 2014b). Rates of brain androgen synthesizing enzyme activity increases following a territorial challenge, where the resident song sparrow is generally considered more dominant (Pradhan et al., 2010), providing a mechanism for neurosteroidal responses. In zebra finches, brain $E_2$ levels vary rapidly based on interaction with potential mates (Remage-Healey et al., 2008). Also, blue-headed wrasse can undergo protogynous behavioral sex change in the absence of gonads (Godwin et al., 1996).

There are several hypotheses to explain why high-ranking females might have lower brain androgen levels. First, high androgen levels are known to have significant metabolic, reproductive, and fitness costs (Ketterson et al., 1992; Wingfield et al., 2001) and thus it is possible that high-ranking females might maintain lower baseline levels to avoid these costs. Second, lower constitutive baseline androgen levels might also allow for transient increases during specific events (Wingfield et al., 1990). In *L. dalli*, while in stable groups, alpha females have lower KT than beta females, however, when the male is removed, and the alpha female rises in status, brain KT increases, but remains unchanged in the beta females (Lorenzi et al., 2012). It must be noted that those differences were seen 24 h after the male was removed from the social group, but in the present experiment, the male was in visual and physical contact with the rest of the group. Third, it is also possible that the lower ranking beta females down regulate androgen receptor expression to reduce the sensitivity to steroid levels, or higher ranking females may have upregulated androgen receptors in the brain, such that sensitivity to steroids may be the
same between alpha and beta females despite differences in steroid levels. The expression of steroid receptors across status classes can be investigated in future studies.

Brain F was elevated in subordinate females compared to dominant females. This result supports the social subordination model, that F is elevated due to stressful events. However, this view does not apply consistently across all classes of vertebrates, probably due to the numerous roles of glucocorticoids in regulating normal physiology (Bonier et al., 2009; Saltzman et al., 1998). Thus it is widely accepted that glucocorticoid levels are largely dependent on the specific mating system in question (Creel, 2001). Finally, ovarian E_2 was higher in alpha females compared to betas and this could be attributed to a greater number of alpha females with a higher ovarian score and more mature ovarian follicles (Figure 6.3).

6.5.4 Interaction among steroid hormones

The bioavailability of steroids for functional effects on phenotypes is regulated at multiple levels, such as steroidogenic enzymes that synthesize or deactivate steroids, binding globulins that prevent steroid metabolism, as well as steroid receptors that bind steroid hormones and initiate activating cellular effects of the steroid (Knapp, 2003). Given that steroidogenic enzymes convert hormones from one form to another and the same enzyme can participate in multiple pathways, the relative amounts of the steroids, rather than the absolute levels might be important to consider. The ratio between two hormones might provide insight into whether one hormone affects another or which steroidogenic enzymes maintain balance and serve as gating mechanism for release of behavior and metabolic processes. Our data show that dominant and subordinate females have differences in ratios in the two tissues we studied here. There are no differences in brain E_2 between alpha and beta females, but the ratios of KT:E_2, T:E_2, and F:E_2 were all different (Fig. 6.7 and Table 6.4), and it is likely that these differences were not driven
by E2. In the ovary, interestingly, T:F was higher in the beta females, even though there was no effect of status in the levels of these steroids (Fig. 6.6, 6.7). In this study, we did not analyze the differences between amounts of each type of hormone, but the ratio data provide those insights without the need for multiple analyses.

From the scales of the axes, it is clear that ovarian E2, is extremely high, compared to KT (Figure 5), but because they are both produced from T, the ratio analysis could be a reasonable method to understand the mechanism of normal ovarian function, as well as during the sex change process. The high ovarian F levels suggest that ovary might sequester F produced in other tissues (such as the inter-renal), or the ovary might synthesize it de novo. Through preliminary in situ hybridization studies, we have found that both isoforms of the enzyme 11β-hydroxysteroid dehydrogenase (one that synthesizes cortisol, and another that deactivates cortisol) are found in the ovary of L. dalli (Pradhan D., Solomon-Lane T., and Grober M., unpublished results). Cortisol has been implicated in affecting several processes in egg production larval maturation by interacting with other hormones. For example, the egg yolk precursor, vitellogenin, the production of which is regulated by E2, is also regulated by F in Arctic char (Berg et al., 2004). Interestingly, while F and E2 administered separately both upregulate vitellogenin in the final step of oogenesis, when administered together, F reduces circulating vitellogenin (Berg et al., 2004). Cortisol also accumulates in eggs, is involved in developmental processes such as larval survival and growth by interacting with thyroid hormones, and reduces in content between fertilization and hatching (reviewed in (Brooks et al., 1997)). Taken together, these data suggest that F plays an important role in reproductive processes and affects tissues differently. In future, ratios could be used to compare the
differences in bioavailability between steroids across different studies that use a variety of sampling and assay methods.

6.5.5 Conclusions

We present data from a novel paradigm that resulted from an opportunistic study looking at social hierarchies in females. These findings on female reproductive morphology and physiology have not been reported previously provide solid groundwork for future studies looking at neurochemical and molecular mechanisms that underlie differences in female social status. Dominant and subordinate female *L. dalli* do not differ in their reproductive maturity (as indicated by GSI), but do have some differences based on ovarian stages. Thus both of these assessments might be complementary when evaluating gonadal contribution to reproductive success. We measured levels of four different steroids, which are seldom reported all together in one study. Tissues of females can be used as a very informative proxy to evaluate steroid levels and endocrine mechanisms.

6.6 Acknowledgements

We thank Madelyne Willis, Megan Williams, Cory Grober, Captain Jack, and Jason Crutcher for help with fieldwork; Kim Schmidt for providing comments on this manuscript; Dr. Anne Murphy for technical support; and the staff at USC Wrigley Institute for Environmental Studies for logistical assistance. We are grateful for the grants from NSERC PGS D3, Sigma Xi, Brains & Behavior Program at Georgia State University and Georgia State University Dissertation Award to DSP, NSF (IOB – 0548567) to MSG, and NSF Doctoral Dissertation Improvement Grant (1210382) to MSG and DSP.
6.7 References


Table 6.1: Morphological Differences between alpha females and beta females in *L. dalli*

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Alpha</th>
<th>Beta</th>
<th>t_{66}</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Length (mm)</td>
<td>35.18 ± 0.31</td>
<td>30.71 ± 0.29</td>
<td>10.54</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body Weight (mg)</td>
<td>730 ± 20</td>
<td>470 ± 10</td>
<td>11.69</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Brain Weight (mg)</td>
<td>5.70 ± 0.23</td>
<td>4.98 ± 0.19</td>
<td>2.42</td>
<td>0.02</td>
</tr>
<tr>
<td>Ovary Weight (mg)</td>
<td>43.07 ± 3.82</td>
<td>32.43 ± 3.63</td>
<td>2.02</td>
<td>0.04</td>
</tr>
<tr>
<td>Ovarian Score</td>
<td>3.27 ± 0.14</td>
<td>2.27 ± 0.14</td>
<td>5.22</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gonadosomatic Index</td>
<td>5.98 ± 0.54</td>
<td>6.80 ± 0.75</td>
<td>0.87</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Mean ± SEM is reported. Alpha females: n=34; Beta females: n=34. Statistically significant p values are in bold.
**Table 6.2**: Effects of male behavior and female status on tissue steroid hormones. Results of within subjects two-way ANOVA are presented.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Brain</th>
<th>Ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F ratio</td>
<td>p</td>
</tr>
<tr>
<td>Testosterone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male nest presence</td>
<td>0.99</td>
<td>0.32</td>
</tr>
<tr>
<td>Female status</td>
<td>25.41</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Male nest presence x female status</td>
<td>0.19</td>
<td>0.67</td>
</tr>
<tr>
<td>11-ketotestosterone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male nest presence</td>
<td>0.03</td>
<td>0.86</td>
</tr>
<tr>
<td>Female status</td>
<td>6.67</td>
<td>0.01**</td>
</tr>
<tr>
<td>Male nest presence x female status</td>
<td>0.12</td>
<td>0.73</td>
</tr>
<tr>
<td>17β-estradiol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male nest presence</td>
<td>1.22</td>
<td>0.27</td>
</tr>
<tr>
<td>Female status</td>
<td>1.75</td>
<td>0.19</td>
</tr>
<tr>
<td>Male nest presence x female status</td>
<td>0.13</td>
<td>0.72</td>
</tr>
<tr>
<td>Cortisol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male nest presence</td>
<td>0.06</td>
<td>0.81</td>
</tr>
<tr>
<td>Female status</td>
<td>8.61</td>
<td>0.005**</td>
</tr>
<tr>
<td>Male nest presence x female status</td>
<td>0.19</td>
<td>0.67</td>
</tr>
</tbody>
</table>

Alpha females: n=34; Beta females: n=34. Statistically significant p values are in bold.
Table 6.3: Effects of alpha female behavior and female status on tissue steroid hormones.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Brain</th>
<th>Ovary</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F ratio</td>
<td>p</td>
<td>F ratio</td>
<td>p</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha female parenting</td>
<td>0.47</td>
<td>0.50</td>
<td>0.40</td>
<td>0.53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female status</td>
<td>25.45</td>
<td><strong>&lt;0.0001</strong></td>
<td>0.04</td>
<td>0.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha female parenting x female status</td>
<td>0.50</td>
<td>0.48</td>
<td>3.73</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11-ketotestosterone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha female parenting</td>
<td>3.17</td>
<td>0.08</td>
<td>0.83</td>
<td>0.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female status</td>
<td>11.49</td>
<td><strong>0.001</strong>*</td>
<td>0.83</td>
<td>0.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha female parenting x female status</td>
<td>0.27</td>
<td>0.61</td>
<td>&lt;0.01</td>
<td>0.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17β-estradiol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha female parenting</td>
<td>0.19</td>
<td>0.19</td>
<td>0.59</td>
<td>0.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female status</td>
<td>0.97</td>
<td>0.33</td>
<td>0.71</td>
<td>0.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha female parenting x female status</td>
<td>0.63</td>
<td>0.43</td>
<td>4.09</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha female parenting</td>
<td>0.21</td>
<td>0.65</td>
<td>&lt;0.01</td>
<td>0.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female status</td>
<td>5.69</td>
<td><strong>0.02</strong>*</td>
<td>0.31</td>
<td>0.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha female parenting x female status</td>
<td>1.27</td>
<td>0.26</td>
<td>1.11</td>
<td>0.24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Alpha females: n=34; Beta females: n=34. Statistically significant p values are in bold.
Table 6.4: Comparison of relative tissue steroid hormone ratios in female *L. dalli*.

<table>
<thead>
<tr>
<th>Steroid Ratio</th>
<th>Brain</th>
<th></th>
<th>Ovary</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U</td>
<td>p</td>
<td>U</td>
<td>p</td>
</tr>
<tr>
<td>KT:T</td>
<td>76.00</td>
<td>0.51</td>
<td>76.00</td>
<td>0.70</td>
</tr>
<tr>
<td>T:E₂</td>
<td>23.50</td>
<td>0.001</td>
<td>0.00</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>T:F</td>
<td>53.00</td>
<td>0.07</td>
<td>33.00</td>
<td>0.006</td>
</tr>
<tr>
<td>KT:F</td>
<td>52.00</td>
<td>0.11</td>
<td>72.00</td>
<td>0.39</td>
</tr>
<tr>
<td>F:E₂</td>
<td>48.00</td>
<td>0.05</td>
<td>83.00</td>
<td>0.75</td>
</tr>
<tr>
<td>KT:E₂</td>
<td>35.00</td>
<td>0.007</td>
<td>73.00</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Alpha females: n=34; Beta females: n=34. Statistically significant p values are in bold.
<table>
<thead>
<tr>
<th>OS</th>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Early in cycle</td>
<td>Lobes are thin, with large inter-lobar space and ample translucent tissue. Eggs are white in color.</td>
</tr>
<tr>
<td>2</td>
<td>Mid-cycle</td>
<td>Lobes are slightly thickened and have decreased translucent tissue. Eggs are whitish-yellow in color.</td>
</tr>
<tr>
<td>3</td>
<td>Late in cycle</td>
<td>Ovaries have increased in volume as yolk granules become visible. Eggs are orange in color.</td>
</tr>
<tr>
<td>4</td>
<td>End of cycle</td>
<td>Ovaries have reached maximum volume with considerably more orange eggs.</td>
</tr>
</tbody>
</table>

**Figure 6.1:** Representative photographs of ovarian function and changes in egg morphology during the breeding season in female *L. dalli* collected off the coast of Santa Catalina Island. Ovarian scores are assigned by stage in ovarian cycle.
Figure 6.2: Frequency distribution of dominant and subordinate female *L. dalli* based on (A) ovarian score and (B) gonadosomatic index. Females were living in stable social groups consisting one male and two size- and status-mismatched females for 4 weeks. Alpha females: n=34 and beta females: n=34.
Figure 6.3: Levels of 17β-estradiol in ovarian tissue categorized by stages of ovarian development (ovarian score) of all alpha and beta female *L. dalli;* n=68. The number inside each bar denotes the sample size of each group. **p<0.01, *p<0.05.
Figure 6.4: Effects of treating male *L. dalli* on baseline and post-treatment (A) agonistic efficiency and (B) duration in nest(s) of all individuals in stable *L. dalli* groups consisting of one male and two status- and size-mismatched females. Males: n=34; Alpha females: n=34; Beta females; ***p<0.001.
Figure 6.5: Levels of brain (A) Testosterone (B) 11-Ketotestosterone (C) 17β-estradiol and (D) Cortisol of female *L. dalli* living in stable social groups consisting one male and two size- and status-mismatched females for 4 weeks. Alpha females (dominant): n=34 and beta females (subordinate): n=34. Alpha females: n=34; Beta females: n=34. Note that the y-axis is different for all hormones. **p<0.01, ***p<0.001.
Figure 6.6: Levels of ovarian (A) Testosterone (B) 11-Ketotestosterone (C) 17β-estradiol and (D) Cortisol of female *L. dalli* living in stable social groups consisting one male and two size- and status-mismatched females for 4 weeks. Alpha females (dominant): n=34; Beta females (subordinate): n=34. Note that the y-axis is different for all hormones. *p<0.05.
Figure 6.7: Comparison of relative tissue hormone ratios (A) Brain and (B) Ovary of dominant and subordinate female *L. dalli*. Alpha females: n=34; Beta females: n=34. **p< 0.01, ***p< 0.001.
7 DISCUSSION

7.1 Breaking biological boundaries: core endocrinological concepts being addressed

Historically, biological molecules that regulate behavior have been categorized based on either location of production, mechanism of action, or structure (Nelson, 2011). My work re-evaluates the validity of those categories, and highlights the artificial nature of boundaries across biological disciplines. It is becoming increasingly clear that modern biology should be integrative and span multiple levels of analyses, ranging from the molecular to systems level.

The current categorization of biological molecules places them in the ‘grey zone’, such that the same molecule can act in more than one manner to enable function. For example, the word hormone has undergone significant changes in meaning over the past several decades (Cawadias, 1940). In modern endocrinology, one concept that has been questioned is the definition that a hormone is secreted by ductless glands, transported by the blood to target organs, and that it takes hours to days for its effects. More recently, over the past decade, it has been established that steroid hormones can act in a much shorter time scale, of seconds to minutes to regulate behavioral changes (Balthazart et al., 2006; Remage-Healey and Bass, 2006). Consequently, the distinctions between hormones and neurotransmitters are quite loose (Adkins-Regan, 2005; Saldanha et al., 2011). The connection between endocrine glands and their secretions being under the influence of the nervous system has been long established (Cawadias, 1940) through the transmission of signals for sex steroid and glucocorticoid synthesis via the hypothalamic–pituitary–gonadal and hypothalamic–pituitary–adrenal axes. However, recent evidence also demonstrates the presence of these axes locally within target tissues, and thus the spatial specificity of steroid transport and action has been revised (Schmidt et al., 2008).
Many different proxies of endocrine measurement are used to determine the relationship between the endocrine system and the regulation of particular reproductive phenotypes. Plasma steroids are most often measured to evaluate phenotypes associated with life history transitions. One of the first studies to show discordance between plasma and brain was performed on rats (Corpéchot et al., 1981). Even though the concepts of spatial effects (location steroid synthesis and action) and temporal effects (time scale of steroid effects) have spurred a re-evaluation of mechanisms by which hormones exert effects, there are tremendous gaps in our knowledge that could be fulfilled by using a comparative species approach.

This dissertation addressed the endocrine and social contexts that regulate life history transitions among a rich suite of phenotypes in a bi-directionally hermaphroditic fish, *Lythrypnus dalli*. I measured reproductive behavior and morphology associated with life history transitions in *L. dalli*. To evaluate the endocrine context and to determine the proximate mechanisms involved in the regulation of phenotypes, I performed systemic and local manipulations and measured the steroid levels. In addition, to get a deeper understanding of social context, I examined components of male and female reproductive behavior in stable and transitioning social groups of fish. The steroid hormones I investigated (testosterone (T), 11-ketotestosterone (KT), 17β-estradiol (E2) and cortisol) are linked through steroidogenic conversion pathways and are known to play a key role in the regulation of reproduction in vertebrate life history transitions. Even though each experiment has its limitations, there was tremendous overlap in the core concepts being evaluated.

In order to develop a comprehensive picture of how biological molecules regulate the expression of reproductive behavior, we need to reevaluate the artificial boundaries. Hypothesis testing should involve experiment design under consideration of both social and environmental
context in which an organism lives (Figure 7.1). Hence it is necessary to study diverse species to provide unique insights on how behavior is controlled more generally. The mechanisms of regulation of behavior have been proposed based on work mainly in rodents, starting with the classical study by Phoenix, Goy, Gerall, and Young (Phoenix et al., 1959). In general, rodent animal models are most prevalent in most laboratories investigating the function and mechanism of hormonal action and the emergent principles apply well in many cases. However, there are many phenomena, especially phenotypic plasticity that cannot be explained by the existing model. There are two major mechanisms that allow for plasticity of phenotype and how this occurs needs to be revaluated. One is the organization and activation of phenotype and second, the regulation of phenotype expression in adulthood. Below, I will discuss the thematic contributions my dissertation work has attempted to make in the field of behavioral neuroendocrinology.

7.2 Organization and activation of phenotype

During development, the embryo is exposed to several types of molecules during critical periods, and together, these organize phenotype of the individual after birth. Many of these organizational features are reflected by the characteristics of sexual phenotype during adulthood (Phoenix et al., 1959). As opposed to mammals, that are canalized to develop into one sex in utero, there are many species that have more flexibility and avoid the fixation of sex. They retain sexual plasticity, whereby, the sex determination is not chromosomal, but environmental (Devlin and Nagahama, 2002). Species in which social environment determines sex, such as many species of fish, activating molecules, such as steroid hormones can play a big role in reorganization of anatomy and behavior. These hormones are critical for maintaining sexually
dimorphic phenotype in most species, and generally males have higher levels of circulating T than females. During protogynous sex change, androgen synthesis and T levels increase, while aromatase activity and E_2 levels decrease. Males of species that show unidirectional sex change, such as wrasses, generally maintain dramatically higher KT levels than females (Perry and Grober, 2003), and this might serve as a mechanism to canalize to the male sexual phenotype. Conversely, in *L. dalli*, species that are capable of lifelong bidirectional sexual plasticity, males and females have similar levels systemic (Lorenzi et al., 2008), brain, and gonadal KT (Lorenzi et al., 2012). Males only show high systemic KT levels when they are actively caring for eggs in their nest, but not when eggs are absent (Rodgers et al., 2006). I hypothesize that this mechanism allows for the capability to rapidly turn off the maintenance of traits or turn on the expression of traits of the opposite sex when the environment is permissive for sex change.

### 7.3 Regulation of phenotype

Based on the environment in which an individual lives, it responds to different contexts accordingly, so as to increase fitness. Local, tissue-specific regulatory systems allow for checkpoints when the signal is globally dispersed (Brenowitz and Lent, 2002). For example, increased T in male rodents allows for the activation of sexual behavior, while gonadectomy gradually eliminates expression of sexual behavior (Damassa et al., 1977). Most of the circulating T in rodents is likely to be gonadally produced and could act as a signal to activate expression of reproductive phenotype through working on multiple different organs that combine to enhance reproductive success. Gonadal T produced during the expression of reproductive behavior is generally very high, however, the quantity of T required to maintain reproductive behavior is considerably low (Damassa et al., 1977). The expression of specific receptors on
either the cellular or nuclear membrane initiates the downstream intracellular events (Nelson, 2011). Some checkpoint may be necessary to buffer the tissue from over stimulation by molecules. It is hypothesized that binding globulins are proteins that bind with hormones to regulate the unnecessary activation of target organs.

Another mechanism that potentially allows maintenance of plasticity, is the dissociation of specific endocrine glands as control centers, and allows specific ‘target’ tissues to control phenotypes. In those cases, the brain can regulate the expression of behavior regardless of signals from the gonad. There are examples from a range of vertebrate, from fish to humans that provide support for local brain regulation of reproductive behavior. First, female wrasses can transform to males when the social environment is permissive, even in the absence of gonads (Godwin et al., 1996). Second, when male L. dalli are intracerebroventricularly (ICV) injected with a KT synthesis inhibitor, their nest care behavior is blocked (Pradhan et al., 2014). However, upon delivery of the inhibitor along with KT rescues those effects (Pradhan et al., 2014). Thus neurally produced KT can regulate male nest care behavior, and there is little evidence for gonadal involvement. Finally, human females can display sexual activity even outside their menstrual cycle. These data evidence for the presence of local control mechanisms, possibly via similar molecular substances in the gonad and brain that can perform similar functions. Downstream cellular mechanisms within the brain that allow for local control of behavior could function via synaptocrine signaling (Saldanha et al., 2011).

### 7.4 Brain steroid synthesis regulates behavior

Steroidogenic enzymes have been identified in the brains of all groups of vertebrates, including fishes, indicating that the brain is capable of producing steroids de novo (Do Rego et
Steroidogenic enzymes in the brain are sensitive to changes in seasons (Pradhan et al., 2010; Riters et al., 2001), life history transformations (Black et al., 2005b), as well as throughout the breeding season when acquiring territories, during courtship, or while parenting (Soma et al., 2003). In *Lythrypnus dalli*, it has been shown previously that both brain and gonadal aromatase activity decrease during female to male sex change (Black et al., 2010; Black et al., 2005b). In Chapter 4, I have shown that activity of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) regulates parenting behavior in *L. dalli*. This enzyme is expressed in fish gonad and brain (Arterbery et al., 2010; Rasheeda et al., 2010), but the direct KT synthesis pathways had not been investigated in depth. *In vitro* regulation of 11β-HSD activity has been investigated to depth in the deactivation of corticosterone (Jellinck et al., 1993; Webb et al., 2008) and thorough studies investigating *in vitro* and *in vivo* regulation of 11β-HSD had been possible through the use of carbenoxolone (CBX) (Jellinck et al., 1993; Latif et al., 1992; Sherbet et al., 2007; Webb et al., 2008), a compound that does not cross the blood brain barrier (Leshchenko et al., 2006). I showed that CBX inhibits 11β-HSD activity *in vitro* in both *L. dalli* gonad and brain. By conducting ICV injection of CBX I showed that a local, tissue-specific manipulation regulates male parenting behavior.

### 7.5 Steroids have rapid effects on behavior

One of the first experiments that showed an effect of steroids on reproductive behavior, was a study on rough-skin newts (Moore and Miller, 1984). Intraperitoneal injection of corticosterone reduced mating behavior within only 2 h (Moore and Miller, 1984). Since then, there have been several studies that have shown that exogenously administered steroids or anti-androgenic or estrogenic chemicals can generate effects on social behavior in a much shorter
time scale than previously thought. Through ICV injection of CBX, I showed that inhibition of
11β-HSD activity reduced male parenting within only 20 min of treatment. Within the same
timeframe, KT rescued parenting when administered along with the inhibitor, while cortisol had
no effects on rates of parenting. During the transitional state and reduced male nest attendance
caused by KT inhibition, dominant females exhibited transient parenting and had elevated brain
KT and reduced ovarian KT. Moreover, these changes were only specific to KT, but not T, E₂,
or cortisol. These results show that rapid behavioral changes are accompanied by tissue-specific
changes in specific steroids, supporting the bi-directional relationship between steroids and
behavior. I also showed in Chapter 2, that intraperitoneal KT implants increased agonistic
behavior within 2 h of the manipulation, but there were no long lasting effects. Together, these
results show that steroids have rapid effects on behavior.

7.6 Androgens do not increase aggressive behavior unless under specific contexts

Many studies have shown that androgen levels increase after aggressive encounters in the
winner of the social challenge (Archer, 2006; Fuxjager et al., 2010; Goymann et al., 2007;
Remage-Healey and Bass, 2005; Wingfield et al., 1990). Similarly, androgen treatment also
causes increases in aggression (Schoech et al., 1998; Weiss and Moore, 2004). However, in
many species, androgens only affect aggression in a context-specific manner, and increased
androgens do not necessarily correspond with increased aggression (Addis et al., 2011; Gill et
al., 2008). In L. dalli, we find little evidence for increased levels of KT also increasing
aggressive behavior proportionally. In Chapter 6, I showed that more dominant females
maintain a higher status by winning agonistic interactions, but have lower levels of KT than
subordinates. However, during periods of social instability, this might not be the case. In the
long-term, males maintain the highest status in the social group; however, there are no sex differences in water-borne and tissue KT levels (Lorenzi et al., 2012; Lorenzi et al., 2008). However, when males care for eggs (while maintaining status), they have higher KT compared to when they are not actively caring for eggs (Rodgers et al., 2006). When subordinate females are implanted with KT (in the absence of a male), they transform into males morphologically, but it does not make them aggressive enough to be dominant to the alpha female and they do not take over the nesting site (Rodgers, 2007). However, I showed in Chapter 2, that when alpha females are implanted with KT (in the absence of a male), they do transform into the male behaviorally and morphologically. Increased rates of aggressive behaviors are not maintained long-term with KT treatment, but only during the critical period of social instability. In Chapter 4, I have shown that brain KT brain injection of KT along with an inhibitor of KT synthesis rescued parenting behavior, while not affecting aggressive behavior. Together, these results show that androgens, such as KT, do not necessarily increase aggression, but rather their effects are dependent upon the social context.

7.7 Androgens and glucocorticoids are not negatively associated with parenting

Androgens and glucocorticoids have generally been viewed negatively because chronically high systemic levels of androgens and glucocorticoids have been associated with adverse effects on parenting in birds (Ketterson and Nolan, 1992; Wingfield et al., 2001) and primates (Gray et al., 2002; Muller et al., 2009; Saltzman and Abbott, 2009). In some rodents and fish, however, high systemic androgens are positively associated with parenting (Marler et al., 2003), and glucocorticoids do not appear to inhibit parenting (Magee et al., 2006; O'Connor et al., 2009). The role of KT in regulating parenting and aggression behavior is very complicated
across species of fish. Even though KT increases rates of aggression in paternal bluegills (Rodgers et al., 2012), in sticklebacks, midshipman fish and blennies, KT does not negatively affect parenting (Knapp et al., 1999; Páll et al., 2002a, b; Ros et al., 2004). The role of systemic cortisol in regulating parenting in fish is not resolved (Magee et al., 2006), but long-term cortisol treatment may increase the rate of nest desertion (O'Connor et al., 2011). Increased glucocorticoids have been associated with reduced reproductive function, such as corticosterone decreases Ledig cell function (Hardy et al., 2005). However the mechanism by which corticosterone mediates these effects are not understood and indirect effects have been proposed through oxidation events through 11β-HSD1. In fish, there is a direct connection between androgens and glucocorticoids through the other isoform, 11β-HSD2. By modulating 11β-HSD in Chapter 4, I found that in the short-term, KT promotes parenting and cortisol does not reduce parenting.

7.8 Behavior of one individual affects other members of the social group

Behavioral changes in one individual can lead to both behavioral and physiological changes in conspecifics (Cheng, 1983; Hutchison and Steimer, 1985). In Chapter 4, I showed that both alpha and beta females opportunistically entered the nest when the male did not establish his territory. Both alpha and beta females who entered the nest consumed eggs, but only alpha females also exhibited parenting. In Chapter 2, I showed that when dominant females showed lower agonistic efficiency, beta females increased their rates of approaches. In Chapter 5, I constructed a transition matrix of various interactions among individuals and showed that when more dominant individuals approach subordinate individuals, the rate of displacement is extremely high. When beta females exhibit solicitation towards males, alpha females interrupt
that solicitation, but beta females do not interrupt the solicitation. These results show that *L. dalli* are very responsive to social context and modify their interactions with conspecifics.

7.9 **Fitness proxies help determine the consequences of neuroendocrine regulation of behavior**

Generally fitness consequences are taken for granted, seldom measured and not taken into consideration when studying mechanisms regulating phenotype. Depending on the life history of a species being investigated, different proxies of fitness can be considered. In the literature, reproductive success refers to the number of offspring produced by an individual, usually within a time period shorter than a lifetime (to distinguish from a direct measure of fitness). In this dissertation, I evaluated parenting behavior of *L. dalli* males in long-term social groups prior to conducting the experiments in Chapter 4. I studied the variation in reproductive behavior and number of eggs produced to determine the relative importance of those behaviors on reproductive success in *L. dalli* and report that in Chapter 5. Through long-term monitoring, I could evaluate the groups where males had sired 2-4 clutches of eggs and use those for my experiment (Chapter 4). During the experiment, I evaluated the fitness consequences of inhibiting male parenting by providing a quantitative measure of egg number. By complementing behavioral measures of females eating eggs with counting the number of eggs, I provide a measure of reproductive success.

7.10 **Future directions**

There are tissue specific differences in levels of steroids in dominant and subordinate females, as well as mini males. I have also determined that neurosteroids regulate parenting by
manipulating in vivo 11β-HSD activity. A major question that remains to be answered is the in vitro regulation of 11β-HSD expression activity during the two critical life history phases: parenting and sex change and also in females of different statuses and mini males. For example, in the dominant female undergoing sexual transformation, brain aromatase activity decreases within hours of male removal, and these changes correlate with increased aggressive behavior (Black et al., 2005a). There are no concurrent changes in gonadal aromatase activity (Black et al., 2005a). These observations, together with our data on circulating hormones suggest that the rate of decrease in brain aromatase activity also corresponds with a decrease in systemic E2 levels. Thus, rapid changes in neurosteroid levels accompany changes in gonadal steroids. It is likely that the decrease in neural aromatase activity increases the neural levels of T as a substrate for conversion to KT (Black et al., 2005a). By examining tissue specific regulation of this enzyme during those transitions and reproductive morphs, we can obtain a better understanding of the biochemical parameters and molecular mechanisms involved. Simultaneous expression of key machinery required for steroid action will provide convergent evidence for regulation of parenting behavior. Targets of steroid action contain machinery, such as steroid receptors, that enable steroids to initiate downstream effects. Preliminary studies have shown sex differences in presence of androgen receptor and 11β-HSD protein in spinal cord and gonads. Thus, I propose to test the hypothesis that 11β-HSD and AR are locally and functionally linked in L. dalli brain to regulate parenting behaviors and I predict that the de novo synthesis of steroids would be coupled with an elevated expression of intracellular steroid receptors. These results will have critical implications for comparative endocrinologists, who have focused almost entirely on gonadally-derived steroids and apply to a wide variety of enzymes based on the species of interest.
Overall, my work provides contextual insights that experience and environment within which an animal lives are important considerations when assessing how and that animal behaves and which cellular mechanisms underlie the expression of phenotype. Even though the exact mechanistic pathways are not yet known, we can highlight some of the issues that need to be investigated in future. Cellular mechanisms to date have been outlined for subsets of vertebrates, mainly based on work in rodents, but those traditional theories do not explain all the variation we see in nature. As a result, we the data that confound the general endocrinological concepts in how molecules work to regulate behavior cannot be ignored and hence a revaluation is necessary to generate a comprehensive model. One way this can be accomplished is by involving more species in basic research, so that a greater diversity can help generate a comprehensive model for the control of reproductive behavior from the systems to biochemical level.

7.11 References


Figure 7.1: Functional interpretation of endocrine studies should be cognizant of the relevant proxy. Loose links exist, such that expression of phenotype is dependent upon both, (A) endocrine and (B) social factors. (C) Balance between these two factors is necessary for the regulation of function and increase lifetime reproductive success. (D) Several endocrine and social context factors must be considered when designing experiments.