Prolactin and Avian Clutch Size: Testing the Only Physiological Mechanism for a Key Life History Trait

by

Calen Patrick Ryan

B.Sc. (Hons.), University of Manitoba, 2010

Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Science

in the
Department of Biological Sciences
Faculty of Science

SIMON FRASER UNIVERSITY

Spring 2013

Approval

Name: **Calen Patrick Ryan**

Degree: **Master of Science**

Title of Thesis: ***Prolactin and Avian Clutch Size: Testing the Only Physiological Mechanism for a Key Life History Trait***

Examining Committee: **Chair:** Dr. Jonathan W. Moore
Assistant Professor

Dr. Tony D. Williams
Senior Supervisor
Professor

Dr. Julian K. Christians
Supervisor
Associate Professor

Dr. Carl A. Lowenberger
Supervisor
Professor

Douglas L. Altshuler
External Examiner
Assistant Professor
Department of Zoology
University of British Columbia

Date Defended/Approved: April 19, 2013

Partial Copyright Licence

Ethics Statement

The author, whose name appears on the title page of this work, has obtained, for the research described in this work, either:

a. human research ethics approval from the Simon Fraser University Office of Research Ethics,

or

b. advance approval of the animal care protocol from the University Animal Care Committee of Simon Fraser University;

or has conducted the research

c. as a co-investigator, collaborator or research assistant in a research project approved in advance,

or

d. as a member of a course approved in advance for minimal risk human research, by the Office of Research Ethics.

A copy of the approval letter has been filed at the Theses Office of the University Library at the time of submission of this thesis or project.

The original application for approval and letter of approval are filed with the relevant offices. Inquiries may be directed to those authorities.

Simon Fraser University Library
Burnaby, British Columbia, Canada

update Spring 2010

Abstract

Clutch size is a fundamental predictor of avian fitness, and has been widely-studied from evolutionary and ecological perspectives. However, surprisingly little is known about the physiological mechanisms that integrate environmental and state-dependant cues into individually-variable responses in clutch size. The only formal mechanistic hypothesis for avian clutch-size determination predicts an anti-gonadal effect of prolactin (PRL) via the inhibition of luteinizing hormone (LH), and has become widely-accepted despite little experimental support. I experimentally tested the PRL-based mechanistic model for clutch size determination in an avian model system, *Taeniopygia guttata* using two complementary approaches. The first involved the pharmacological manipulation of PRL, while the second entailed experimentally enlarging clutch size. Contrary to predictions based on the PRL-based mechanistic model, PRL, LH and clutch size were effectively uncoupled through experimental manipulation. These findings expose a serious gap in our understanding of the physiological mechanisms of clutch size, a key life history trait.

Keywords: life history; physiological mechanisms; avian reproduction; plasticity; prolactin; clutch size

Dedication

This thesis is dedicated to: P.L.R. for her courage, strength, and perseverance; T.R.R., for his sharp, critical, and inquisitive mind; M.S.R., for her creativity, pragmatism, and astonishing work ethic, and; K.A.V. for her unending support and wisdom. I also owe this thesis to M.D. for her patience and forgiveness, A.C.C. for her fearlessness and undying confidence in me, N.D. for her enthusiasm and creativity, S.H for her illumination and friendship, J.A. for his humour and priorities, S.I. for his open-mind and inquisitiveness, and my crew at SFU Grappling and Titan Mixed Martial Arts for humbling me and building me at the same time.

Acknowledgements

I am deeply grateful to my supervisor, Professor Tony D. Williams for giving me the freedom to explore my curiosities while reigning in my meanderings, and for challenging and expanding my views of individual variation, hormones, and life history evolution. Similarly, I owe part of my success to the feedback of my lab-mates, whose keen and honest criticism unquestionably improved my work. Laramie Ferguson and Hong Ho provided keen and meticulous volunteer assistantship with the zebra finches. I am also indebted to Drs. Julian K. Christians and Carl A. Lowenberger for their careful scrutiny and insightful suggestions, and to Dr. Douglas Altshuler for his excellent job as external examiner. All three of these gentlemen provided insightful and stimulating comments and questions to my work. I am thankful to Drs. Alistair Dawson and Peter Sharp for accepting me into their labs in Edinburgh, Scotland, and providing me with the supplies and guidance to conduct my hormone assays. I am also grateful to Dr. Simone Meddle for her ongoing support at the Roslin Institute. Professor Ton Groothuis and his lab kindly hosted me in Groningen, Netherlands, and provided helpful comments at the early stages of my data analysis. Last, but not least, the Natural Sciences and Engineering Research Council of Canada and the Simon Fraser University, Dean of Graduate Studies, provided funding, without which this project would not have been possible.

Table of Contents

List of Figures

List of Acronyms

LH	Immunoactive luteinizing hormone
PRL	Immunoactive prolactin
E2	Estrogen
P4	Progesterone
T	Testosterone
PRL-R	Prolactin receptor
GnRH	Gonadotropin-releasing hormone
DA	Dopamine
D2	Type-2 dopamine receptor

Chapter 1.

General Introduction

1.1. Introduction

Lifetime reproductive success, or fitness, is largely dependent on the frequency, timing, and magnitude of investment into growth, survival and reproduction (Stearns 1992; Roff 1992). In turn, these three categories of investment compete with each other for finite time, energy, and resources, resulting in trade-offs that constrain their optimal expression (Stearns 1989). Similarly, allocation of time or resources to one component of reproduction often necessitates costs to another (e.g. offspring number versus size; Harshman and Zera 2007). The trade-offs between and within growth, survival, and reproduction are built upon their evolutionary legacies and are shaped by the ecological conditions in which they are expressed (Roff 1992). Over time, strategies for investment into these three broad categories combine to create trait complexes that tend towards maximizing fitness within a given ecological context or range of contexts (Flatt and Heyland 2011). These assemblages, or 'life histories', have become integral to the study of evolution, ecology, and behaviour (e.g. Partridge and Harvey 1988; Réale et al. 2000).

Broad-scale ecological and phylogenetic patterns in life history traits are well-documented (Berven and Gill 1983; Böhning-Gaese and Oberrath 1999; Martin et al. 2006), but these patterns are also overlain by marked intra-specific variability in life histories (Cam et al. 2002; Tuljapurkar and Steiner 2010). For example, neighbouring populations of the same species may show distinct variability in life history traits (Caro et al. 2005; Garant et al. 2007), as may genetically similar individuals under controlled laboratory conditions (Barata and Baird 1998; Earley et al. 2012). In addition to inter-individual variation in life history traits, considerable intra-individual plasticity and variability in life history traits is also common. A female bird, for example, may modify the

timing and magnitude of investment into reproduction (e.g. egg number or provisioning effort) between breeding seasons, decisions that can have significant individual fitness consequences (Boyce and Perrins 1987; McCleery et al. 2004; Maccoll and Hatchwell 2004). Thus, individual variability in life history traits provides the raw material for selection, while intra-individual plasticity and variability in life history traits also allow individuals to fine-tune their investment strategically (McNamara and Houston 1996).

At the scale of the individual, life history traits generally involve complex developmental, physiological, and behavioural components (Finch and Rose 1995). The nature, timing, and magnitude of these phenotypic traits must be tightly coordinated with each other and with both internal and external cues (Denver et al. 2009; Martin et al. 2011). For example, a female bird is required to coordinate her physiological preparedness to reproduce with the seasonal availability of food and presence of a male, as well as with a behavioural readiness to incubate and care for young. Thus, in the short-term, individuals integrate ecological, social, and state-dependant physiological information and respond with alterations to numerous phenotypic traits (e.g. behavioural, physiological etc.) at biologically very different scales (e.g. cells, tissues, organs etc.; Zera et al. 2007).

While life history theory has been widely-studied in evolutionary and ecological frameworks, the underlying physiological and neuroendocrinological mechanisms that integrate internal and external cues into coordinated, and potentially adaptive, life history responses are only rudimentarily understood (Finch and Rose 1995; Zera and Harshman 2001; Flatt and Heyland 2011). Even less is known about individual plasticity in neuroendocrine mechanisms and how they relate to individual plasticity in life history traits (Lessells 2008; Williams 2008). Nonetheless, understanding the mechanistic underpinnings of life history traits is crucial because physiological mechanisms can impose costs or constraints that can limit the evolution of those traits, or the capacity for species or individuals to respond to environmental change (Wingfield et al. 2008; Cohen et al. 2012).

The study of avian systems has had a significant impact on our understanding of life history theory (Lack 1947; Martin 2004; Wingfield et al. 2008; Ricklefs 2010). Early empirical findings and theoretical predictions have been followed up by long-term

datasets measuring fitness correlates of avian life history traits (McCleery et al. 2004) and plasticity or 'reaction norms' in those traits (Brommer et al. 2005). While a staggering array of hypotheses about the ecological factors and trade-offs that drive life history traits and transitions in birds have been generated (e.g. see Figs. 2 and 3 in Martin 2004), the lack of experimental work adequately testing these hypotheses has been a source of criticism (Martin 2004). Notable exceptions include 'phenotypic engineering' studies in dark-eyed Juncos (*Junco hyemalis*), which have illuminated the role of testosterone (T) in trade-offs between survival and reproduction, and mating effort and parental investment (Ketterson et al. 1996). Thus, while studying physiological and endocrinological mechanisms can provide data essential for testing both evolutionary and ecological hypotheses about avian life history, examples of such approaches in the literature are still rare (Zera and Harshman 2001; Williams 2012b).

One of the more obvious examples of such oversight is in the study of avian clutch size. Clutch size is one of the most important life history traits with respect to fitness in female birds (McCleery et al. 2004), and arguably has been studied from evolutionary and ecological perspectives more than any other avian life history trait (e.g. Lack 1947; Cody 1966; Rowe et al. 1994; Martin 2004). However, despite a long history of interest in the physiological mechanisms for clutch size determination (Klomp 1970), there has been surprisingly little serious investigation in this area (Williams 2012a). For some time, prolactin (PRL), a peptide hormone with important functions in incubation and parental care (Sharp et al. 1998), has been suggested to play a role in clutch size determination in birds (Murton and Westwood 1977; Haftorn 1981; Lea et al. 1981). Early speculations about a regulatory role of PRL in clutch size determination were subsequently supported by a growing body of circumstantial evidence that over time have evolved into a formal mechanistic hypothesis with explicit predictions (Meijer et al. 1990), eventually incorporating the seasonal timing of breeding, hatching asynchrony, and maternal effects in the form of yolk androgen deposition (Sockman et al. 2006). Additional claims have been made based partly on the presumed validity of this hypothetical mechanism, such as a recent report that pre-breeding PRL in house sparrows (*Passer domesticus*) are predictive of fledging number and reproductive success (Ouyang et al. 2011). Yet even in its more basic forms (Meijer et al. 1990; Haywood 1993), the PRL-based mechanistic model for avian clutch size determination

has very little empirical support, especially from experimental studies. Equivocal results in the one experimental study to date addressing this problem directly (Sockman et al. 2000) unfortunately left the chief question largely unanswered: Does circulating PRL play a mechanistic role in avian clutch size determination?

The main goal of my thesis was to test experimentally the PRL-based mechanistic model in its simplest form, i.e. to test the hypothesis that circulating PRL during laying acts as a regulatory factor in avian clutch size determination, using captive breeding zebra finches (*Taeniopygia guttata*) as an avian model system. Because PRL has also been proposed to inhibit follicular recruitment or growth indirectly through the suppression of luteinizing hormone (LH; Lea et al. 1981), I also examined the relationship between PRL, LH, and clutch size. Two complementary approaches were employed to uncouple the trait, clutch size, from its putative regulatory factor, PRL. The first approach (Chapter 2) involved the pharmacological manipulation of PRL using the dopamine receptor-2 agonist, bromocriptine. Bromocriptine binds to the inhibitory D2 receptor on PRL-secretory lactotroph cells in the pituitary, and has been widely used to lower PRL in mammals (e.g. Bridges and Ronsheim 1990; Roberts et al. 2001), and less commonly, in birds (Jouventin and Mauget 1996; Angelier et al. 2006). The main goal of this study was to reduce circulating PRL levels during laying and examine the effects of this manipulation on subsequent clutch size. We also looked at PRL and LH in pre-breeding zebra finches, to test whether or not these hormones are predictive of clutch size in a subsequent breeding attempt. This study focused mainly on general hormonal patterns and changes in PRL and LH between pre-breeding and breeding states, as well as individual changes between the control breeding and bromocriptine breeding. The second approach (Chapter 3), complementary to the first, involved manipulating clutch size itself through the continual removal of eggs as they were laid. The main goal for this study was to increase clutch size, and subsequently examine changes in PRL and LH. This chapter explores the relationship between PRL and clutch size in greater detail, expanding on the concept of hormonal fluctuations and co-variation in PRL, LH, and clutch size through time. For both studies, condition-associated traits like body size and mass were examined alongside PRL and LH. We also measured egg size, laying interval, and mass loss during breeding along with clutch size, to quantify relative reproductive investment. Both of these studies were carried out in the same females, for

whom we possessed detailed information on age, growth, and reproductive history. By tracking individual females through pre-breeding, control and experimental breeding attempts, we were able to study individual plasticity in reproductive traits as well as individual response to treatment.

1.2. References

Angelier, Frédéric, Christophe Barbraud, Hervé Lormée, François Prud'homme, and Olivier Chastel. 2006. "Kidnapping of chicks in emperor penguins: a hormonal by-product?" *Journal of Experimental Biology* 209 (8) (April 15): 1413 –1420. doi:10.1242/jeb.02138.

Barata, C., and D. J. Baird. 1998. "Phenotypic plasticity and constancy of life-history traits in laboratory clones of Daphnia magna Straus: effects of neonatal length." *Functional Ecology* 12 (3): 442–452. doi:10.1046/j.1365-2435.1998.00201.x.

Berven, Keith A., and Douglas E. Gill. 1983. "Interpreting Geographic Variation in Life-History Traits." *American Zoologist* 23 (1) (January 1): 85–97. doi:10.1093/icb/23.1.85.

Böhning-Gaese, K, and Reik Oberrath. 1999. "Phylogenetic effects on morphological, life-history, behavioural and ecological traits of birds." *Evolutionary Ecology Research* 1: 347–364.

Boyce, Mark S., and C. M. Perrins. 1987. "Optimizing Great Tit Clutch Size in a Fluctuating Environment." *Ecology* 68 (1) (February 1): 142–153. doi:10.2307/1938814.

Bridges, Robert S., and Paul M. Ronsheim. 1990. "Prolactin (PRL) Regulation of Maternal Behaviour in Rats: Bromocriptine Treatment Delays and PRL Promotes the Rapid Onset of Behaviour." *Endocrinology* 126 (2) (February 1): 837 –848. doi:10.1210/endo-126-2-837.

Brommer, Jon E., Juha Merilä, Ben C. Sheldon, Lars Gustafsson, and P. Phillips. 2005. "Natural selection and genetic variation for reproductive reaction norms in a wild bird population." *Evolution* 59 (6): 1362–1371. doi:10.1554/04-561.

Cam, Emmanuelle, William A. Link, Evan G. Cooch, Jean-Yves Monnat, and Etienne Danchin. 2002. "Individual Covariation in Life-History Traits: Seeing the Trees Despite the Forest." *The American Naturalist* 159 (1) (January): 96–105. doi:10.1086/324126.

Caro, Samuel P., Jacques Balthazart, Donald W. Thomas, André Lacroix, Olivier Chastel, and Marcel M. Lambrechts. 2005. “Endocrine correlates of the breeding asynchrony between two corsican populations of blue tits (*Parus caeruleus*).” *General and Comparative Endocrinology* 140 (1) (January 1): 52–60. doi:10.1016/j.ygcen.2004.09.016.

Cody, Martin L. 1966. “A general theory of clutch size.” *Evolution*: 174–184.

Cohen, Alan A., Lynn B. Martin, John C. Wingfield, Scott R. McWilliams, and Jennifer A. Dunne. 2012. “Physiological regulatory networks: ecological roles and evolutionary constraints.” *Trends in Ecology & Evolution* 27 (8) (August): 428–435. doi:10.1016/j.tree.2012.04.008.

Denver, Robert J., Penny M. Hopkins, Stephen D. McCormick, Catherine R. Propper, Lynn Riddiford, Stacia A. Sower, and John C. Wingfield. 2009. “Comparative endocrinology in the 21st century.” *Integrative and Comparative Biology* 49 (4) (October 1): 339–348. doi:10.1093/icb/icp082.

Earley, Ryan L., Amanda F. Hanninen, Adam Fuller, Mark J. Garcia, and Elizabeth A. Lee. 2012. “Phenotypic Plasticity and Integration in the Mangrove Rivulus (*Kryptolebias marmoratus*): A Prospectus.” *Integrative and Comparative Biology* 52 (6) (December 1): 814–827. doi:10.1093/icb/ics118.

Finch, Caleb E., and Michael R. Rose. 1995. “Hormones and the physiological architecture of life history evolution.” *The Quarterly Review of Biology* 70 (1) (March 1): 1–52. doi:10.2307/3037130.

Flatt, Thomas, and Andreas Heyland. 2011. *Mechanisms of life history evolution: the genetics and physiology of life history traits and trade-offs*. Oxford University Press, May 12.

Garant, Dany, Loeske E. B. Kruuk, Robin H. McCleery, and Ben C. Sheldon. 2007. “The Effects of Environmental Heterogeneity on Multivariate Selection on Reproductive Traits in Female Great Tits.” *Evolution* 61 (7): 1546–1559. doi:10.1111/j.1558-5646.2007.00128.x.

Haftorn, Svein. 1981. “Incubation during the Egg-Laying Period in Relation to Clutch-Size and Other Aspects of Reproduction in the Great Tit *Parus major*.” *Ornis Scandinavica* 12 (3) (December 1): 169–185. doi:10.2307/3676074.

Harshman, Lawrence G., and Anthony J. Zera. 2007. “The cost of reproduction: the devil in the details.” *Trends in Ecology & Evolution* 22 (2) (February): 80–86. doi:10.1016/j.tree.2006.10.008.

Haywood, S. 1993. “Sensory and Hormonal-Control of Clutch Size in Birds.” *Quarterly Review of Biology* 68 (1) (March): 33–60.

Jouventin, P., and R. Mauget. 1996. “The endocrine basis of the reproductive cycle in the king penguin (*Aptenodytes patagonicus*).” *Journal of Zoology* 238 (4): 665–678. doi:10.1111/j.1469-7998.1996.tb05421.x.

Ketterson, E. D., V. Nolan, M. J. Cawthorn, P. G. Parker, and C. Ziegenfus. 1996. "Phenotypic engineering: Using hormones to explore the mechanistic and functional bases of phenotypic variation in nature." *Ibis* 138 (1) (January): 70–86.

Klomp, H. 1970. "The determination of clutch-size in birds: a review." *Ardea*.

Lack, David. 1947. "The Significance of Clutch-size." *Ibis* 89 (2): 302–352. doi:10.1111/j.1474-919X.1947.tb04155.x.

Lea, R. W., A. S. M. Dods, P. J. Sharp, and A. Chadwick. 1981. "The Possible Role of Prolactin in the Regulation of Nesting Behaviour and the Secretion of Luteinizing Hormone in Broody Bantams." *Journal of Endocrinology* 91 (1) (October 1): 89–97. doi:10.1677/joe.0.0910089.

Lessells, C(Kate) M. 2008. "Neuroendocrine control of life histories: what do we need to know to understand the evolution of phenotypic plasticity?" *Philosophical Transactions of the Royal Society B: Biological Sciences* 363 (1497) (May 12): 1589–1598. doi:10.1098/rstb.2007.0008.

Maccoll, Andrew D. C., and Ben J. Hatchwell. 2004. "Determinants of lifetime fitness in a cooperative breeder, the long-tailed tit *Aegithalos caudatus*." *Journal of Animal Ecology* 73 (6): 1137–1148. doi:10.1111/j.0021-8790.2004.00887.x.

Martin, Lynn B., Andrea L. Liebl, Justin H. Trotter, Christina L. Richards, Krista McCoy, and Michael W. McCoy. 2011. "Integrator Networks: Illuminating the Black Box Linking Genotype and Phenotype." *Integrative and Comparative Biology* (June 25). doi:10.1093/icb/icr049.

Martin, Thomas E. 2004. "Avian life-history has an eminent past: does it have a bright future?" *The Auk* 121 (2): 289.

Martin, Thomas E., R. D. Bassar, S. K. Bassar, J. J. Fontaine, P. Lloyd, H. A. Mathewson, A. M. Niklison, and A. Chalfoun. 2006. "Life-history and ecological correlates of geographic variation in egg and clutch mass among passerine species." *Evolution* 60 (2): 390–398. doi:10.1111/j.0014-3820.2006.tb01115.x.

McCleery, R.H., R.A. Pettifor, P. Armbruster, K. Meyer, B.C. Sheldon, and C. Perrins. 2004. "Components of Variance Underlying Fitness in a Natural Population of the Great Tit *Parus major*." *The American Naturalist* 164 (3) (September 1): E62–E72.

McNamara, John M., and Alasdair I. Houston. 1996. "State-dependent life histories." *Nature* 380 (6571) (March 21): 215–221. doi:10.1038/380215a0.

Meijer, Theo, Serge Daan, and Michael Hall. 1990. "Family Planning in the Kestrel (*Falco tinnunculus*): The Proximate Control of Covariation of Laying Date and Clutch Size." *Behaviour* 114 (1/4): 117–136.

Murton, R.K., and N.J. Westwood. 1977. *Avian breeding cycles*. Oxford: Clarendon Press.

Ouyang, Jenny Q., Peter J. Sharp, Alistair Dawson, Michael Quetting, and Michaela Hau. 2011. “Hormone levels predict individual differences in reproductive success in a passerine bird.” *Proceedings of the Royal Society B: Biological Sciences* (January 19). doi:10.1098/rspb.2010.2490.

Partridge, Linda, and Paul H. Harvey. 1988. “The Ecological Context of Life History Evolution.” *Science* 241 (4872) (September 16): 1449–1455. doi:10.1126/science.241.4872.1449.

Réale, Denis, Bruno Y. Gallant, Mylène Leblanc, and Marco Festa-Bianchet. 2000. “Consistency of temperament in bighorn ewes and correlates with behaviour and life history.” *Animal Behaviour* 60 (5) (November): 589–597. doi:10.1006/anbe.2000.1530.

Ricklefs, Robert E. 2010. “Parental Investment and Avian Reproductive Rate: Williams’s Principle Reconsidered.” *The American Naturalist* 175 (3) (March 1): 350–361.

Roberts, R L, K T Jenkins, T Lawler Jr, F H Wegner, and J D Newman. 2001. “Bromocriptine administration lowers serum prolactin and disrupts parental responsiveness in common marmosets (*Callithrix j. jacchus*).” *Hormones and Behaviour* 39 (2) (March): 106–112. doi:10.1006/hbeh.2000.1639.

Roff, Derek A. 1992. *Evolution of life histories: theory and analysis*. Springer.

Rowe, L., D. Ludwig, and D. Schluter. 1994. “Time, condition, and the seasonal decline of avian clutch size.” *The American naturalist* 143 (4): 698–722.

Sharp, Peter J., Alistair Dawson, and Robert W. Lea. 1998. “Control of luteinizing hormone and prolactin secretion in birds.” *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology* 119 (3) (June): 275–282. doi:10.1016/S0742-8413(98)00016-4.

Sockman, K. W., H. Schwabl, and P. J. Sharp. 2000. “The role of prolactin in the regulation of clutch size and onset of incubation behaviour in the American kestrel.” *Hormones and behaviour* 38 (3) (November): 168–176.

Sockman, Keith W., Peter J. Sharp, and Hubert Schwabl. 2006. “Orchestration of avian reproductive effort: an integration of the ultimate and proximate bases for flexibility in clutch size, incubation behaviour, and yolk androgen deposition.” *Biological Reviews* 81 (04) (October): 629. doi:10.1017/S1464793106007147.

Stearns, S. C. 1989. “Trade-offs in life-history evolution.” *Functional ecology* 3 (3): 259–268.

Stearns, Stephen C. 1992. *The Evolution of Life Histories*. Oxford University Press, USA, July 16.

Tuljapurkar, Shripad, and Ulrich K. Steiner. 2010. “Dynamic heterogeneity and life histories.” *Annals of the New York Academy of Sciences* 1204 (1): 65–72. doi:10.1111/j.1749-6632.2010.05519.x.

Williams, Tony D. 2008. “Individual variation in endocrine systems: moving beyond the ‘tyranny of the Golden Mean’.” *Philosophical Transactions of the Royal Society B: Biological Sciences* 363 (1497) (May 12): 1687 –1698. doi:10.1098/rstb.2007.0003.

Williams, Tony D. 2012a. “Hormones, life-history, and phenotypic variation: Opportunities in evolutionary avian endocrinology.” *General and Comparative Endocrinology* (0). doi:10.1016/j.ygcen.2011.11.028. http://www.sciencedirect.com/science/article/pii/S0016648011004424.

———. 2012b. *Physiological Adaptations for Breeding in Birds*. Princeton, NJ: Princeton University Press, August 5.

Wingfield, John C, Tony D Williams, and Marcel E Visser. 2008. “Introduction. Integration of ecology and endocrinology in avian reproduction: a new synthesis.” *Philosophical Transactions of the Royal Society B: Biological Sciences* 363 (1497) (May 12): 1581 –1588. doi:10.1098/rstb.2007.0012.

Zera, A. J., and L. G. Harshman. 2001. “The physiology of life history trade-offs in animals.” *Annual Review of Ecology and Systematics* 32: 95–126.

Zera, Anthony J., Lawrence G. Harshman, and Tony D. Williams. 2007. “Evolutionary Endocrinology: The Developing Synthesis between Endocrinology and Evolutionary Genetics.” *Annual Review of Ecology, Evolution, and Systematics* 38 (1) (December): 793–817. doi:10.1146/annurev.ecolsys.38.091206.095615.

Chapter 2.

Individual Variation in Pre-Breeding and Breeding Prolactin and Luteinizing Hormone in Relation to Clutch Size

Calen P. Ryan[1], Alistair S. Dawson[2], Peter J. Sharp[3], and Tony D. Williams[1]

[1]Department of Biological Sciences, Simon Fraser University, 8888 University Drive, Burnaby, British Columbia, Canada, V5A 1S6; [2]Centre for Ecology and Hydrology, Bush Estate, Penicuik, Midlothian, Scotland, U.K., EH26 0QB; [3]The Roslin Institute, University of Edinburgh, Easter Bush, Midlothian, Scotland, U.K., E25 9RG

2.1. Introduction

Avian clutch size sets the upper limit for the number of successfully fledged chicks and is a key predictor of reproductive investment and lifetime reproductive success (Rockwell et al. 1987; McCleery et al. 2004; Charmantier et al. 2006). Explaining the patterns and variability in clutch size within and among avian populations has long been a goal of both evolutionary biologists and ecologists (Lack 1947; Williams 1966; Klomp 1970; Godfray et al. 1991; Ricklefs 2010). Much of the effort has gone into understanding the evolutionary forces and constraints that shape optimal clutch size (e.g. Rockwell et al. 1987; Pettifor et al. 1988; Lessells et al. 1989; Nager et al. 2000), or the ecological cues individuals employ to fine tune clutch size (e.g. Travers et al. 2010; Zanette et al. 2011; Sibly et al. 2012; Decker et al. 2012). However, understanding the physiological mechanisms that coordinate key life history traits and transitions is paramount to resolving their ecological limitations and evolutionary legacies (Ricklefs and Wikelski 2002; Williams 2012a). Nonetheless, the fundamental physiological and hormonal mechanisms that coordinate clutch size and other important life history traits remain poorly understood (Klomp 1970; Sockman et al. 2006; Williams 2012b).

The only physiological or mechanistic hypothesis for avian clutch size determination involves an anti-gonadal effect of prolactin (PRL), a peptide hormone that stimulates, and is stimulated by, incubation behaviour (El Halawani et al. 1984; March et al. 1994; Delehanty et al. 1997) and chick rearing (O'Dwyer et al. 2006; Angelier and Chastel 2009; Miller et al. 2009). This hypothesis is built upon a number of well-supported observations, namely: a) the close temporal association between the development of incubation behaviour and the cessation of laying (Lea et al. 1981; Haftorn 1981); b) a positive feedback loop between the development of incubation behaviour, tactile stimulation from the eggs, and plasma PRL levels (El Halawani et al. 1984; Massaro et al. 2007), and; c) the seasonal increase in rate of incubation onset accompanied by a seasonal decline in clutch size (Meijer et al. 1990; Flint et al. 2006). Along with a putative anti-gonadal effect of PRL (discussed below), these observations provide support for a mechanistic model whereby PRL regulates clutch size, and is itself mediated through seasonal increases in PRL and the onset of incubation behaviour (Murton and Westwood 1977; Haftorn 1981; Meijer et al. 1990; Haywood 1993;

Sockman et al. 2006). Much of the data used to support the PRL-based mechanism for clutch size determination is based on broad temporal correlations rather than direct experimental evidence, and there remains little support for: a direct association between clutch size and plasma PRL 2-4 days after first laid egg, the temporal window when follicular inhibition of clutch size determination is thought to occur in several species, and; an anti-gonadal effect of PRL sufficient to cause follicular inhibition and the cessation of laying. Potential anti-gonadal effects of PRL via inhibition of gonadotropin releasing hormone (GnRH) I and II and luteinizing hormone (LH) have been demonstrated in localized *in vitro* assays in poultry (El Halawani et al. 1984; Rozenboim et al. 1993; You et al. 1995), and are supported by evidence for anti-gonadal effects of PRL *in vivo* in some species (Bailey 1950; Meier 1969; Reddy et al. 2007). However, an anti-gonadal effect of PRL has not been found in other species (Meier and Dusseau 1968; Buntin et al. 1999; Small et al. 2007), and whether or not experimentally manipulated PRL levels, let alone natural individual variation in PRL, have the capacity to inhibit or disrupt follicle growth and directly affect clutch size has not yet been demonstrated.

The only experimental work to examine circulating PRL and clutch size determination directly in a non-domesticated species laying discrete clutches (i.e. retaining cyclic reproduction characteristic of wild birds) was carried out by Sockman et al. (2000) in the American Kestrel, *Falco sparverius*. This study found weak support for a negative association between clutch size and PRL at the time when follicular inhibition putatively occurs. However, PRL manipulations using ovine-PRL osmotic minipumps were unsuccessful and were not associated with changes in clutch size (Sockman et al. 2000). Based on these results, the authors themselves emphasized in a later review that "*a role for prolactin in regulating clutch size in any species is not firmly established*", and that further work in this area is necessary (Sockman et al. 2006). Despite the prudent conclusions Sockman and his colleagues, the PRL-based model for clutch size determination has received little attention since, despite the importance of clutch size to avian reproductive success (McCleery et al. 2004; Williams 2012a).

The PRL-based model of clutch size determination (Meijer et al. 1990; Haywood 1993) focuses on variation in circulating PRL levels 2-4 days after the first egg is laid, when follicular inhibition and clutch size determination is thought to occur. However,

several recent studies have suggested that pre-breeding hormone levels might influence, or potentially predict, subsequent reproductive performance (Chastel et al. 2003; Greives et al. 2012; Crossin et al. 2012). For example, in a study of free-living house sparrows (*Passer domesticus*), pre-laying PRL levels were correlated with fledging success, although this effect was highly dependant on the effect of lay date (Ouyang et al. 2011). Alternatively, Schaper et al. (2012) suggested that pre-breeding PRL levels may be a stronger indicator of seasonal reproductive readiness than an accurate proxy for breeding investment in the form of clutch size. Whether or not pre-breeding PRL is predictive of subsequent reproductive performance (in particular, clutch size) under controlled environment and photoperiod has, to our knowledge, not been examined.

Here we investigate individual variability in plasma PRL and LH in pre-breeding and breeding females in relation to individual variation in clutch size in the zebra finch, *Taeniopygia guttata,* to test predictions from the PRL-based mechanism of clutch size determination (Murton and Westwood 1977; Haftorn 1981; Meijer et al. 1990; Haywood 1993). We used a repeated-measures design to follow individuals of known age and reproductive history through pre-breeding, breeding, and experimental breeding states under controlled environmental and photoperiodic conditions. Our specific objectives were to determine: 1) the relationship between measures of condition (e.g. mass, hematocrit) and changes in plasma PRL and LH between pre-breeding and breeding states in individual females; 2) the relationship between pre-breeding hormones and subsequent clutch size, and; 3) the relationship between plasma PRL and LH levels sampled at the putative time of clutch size determination in zebra finches (the day the 3rd egg was laid; Haywood 1993) and clutch size. We also attempted to experimentally decrease plasma PRL levels using the dopamine receptor agonist bromocriptine (Badyaev and Duckworth 2005; Angelier et al. 2006; Reddy et al. 2007), thereby disrupting the putative endogenous relationship between PRL and clutch size. Based on the PRL-based mechanism of clutch size determination, we predicted: a) a negative correlation between PRL and LH; b) a negative association between breeding plasma PRL levels and clutch size, and; c) an increase in clutch size associated with a decrease in PRL in bromocriptine-treated females.

2.2. Methods and Materials

2.2.1. Animal care and breeding protocol

Zebra finches were maintained in controlled environmental conditions (temperature 19–23°C; humidity 35–55%; constant light schedule, 14 L: 10 D, lights on at 07.00). All birds were provided with a mixed seed diet (*Panicum* and white millet, 1:3, 11.7% protein, 0.6% lipid and 84.3% carbohydrate by dry mass), water, grit and cuttlefish bone (calcium) *ad libitum*, and received a multi-vitamin supplement in the drinking water once per week. Breeding pairs were also provided with 6 g/pair/day of egg food supplement (20.3% protein, 6.6% lipid) between pairing and clutch completion.

Before the experiment, all birds were housed in same-sex cages (61cm x 46cm x 41cm) but were not visually or acoustically isolated from the opposite sex. Individual females used in experiments were 4-8 months of age, had been successfully bred at least once, and were always paired with the same male to minimize variation in investment based on perceived mate quality. Breeding pairs were housed individually in single cages (61cm x 46 cm x 41 cm), each with an external nest-box (11.5cm x 11.5cm x 11.5cm). Females were weighed (± 0.1 g, initial mass) at the time of pairing, just prior to blood sampling, and at clutch completion. During breeding, nest-boxes were checked daily between 09.30 and 11.30 and all new eggs were weighed (to 0.001 g) and numbered, to obtain data on egg size, clutch size and laying interval (the time between pairing and laying of the first egg). A clutch was considered complete when no additional eggs were produced over two consecutive days. At clutch completion, eggs were removed and individuals were returned to same-sex holding cages for a recovery period of at least three weeks. Experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (no. 901B 94), in accordance with guidelines from the Canadian Committee on Animal Care (CCAC).

2.2.2. Blood sampling and hormone analysis

Females were blood sampled (≤200 µL, from the brachial vein) prior to breeding while in same-sex holding cages (‘pre-breeding’), and following pairing (females paired 13-17 days later) on the day the third egg was laid (‘breeding’). Egg day three was

selected based the experimental work which links the physiological mechanism for clutch size determination in Zebra Finches with the timing of the third laid egg (Haywood 1993). Blood samples for the bromocriptine experiment were also taken on the day the third egg was laid. Blood sampling was carried out between 1130 and 1330 to minimize circadian fluctuations in hormone levels, blood was centrifuged at 13,000 rpm for five minutes, and plasma was stored at –20°C until required for hormone assays.

Plasma immunoreactive prolactin (PRL) was determined using a radioimmunoassay for recombinant-derived Starling (*Sturnus vulgaris)* PRL described by Bentley et al. (1997). Other than two blood samples for which there was insufficient plasma, all samples were measured in duplicate in a single assay, diluted 1 in 3. The sensitivity of the assay, determined to be the estimated concentration two standard deviations above the mean counts per minute of the lowest standard, was 7.779 $ng \cdot mL^{-1}$, after correcting for dilution. Samples with concentrations below this value were not included in analyses unless stated otherwise. The intra-assay coefficient of variation of this assay was 6.5%, and serial dilution of individual samples ran parallel along the standard curve within the range assayed. Luteinizing hormone (LH) was measured using a micro-modified version of a previously described radioimmunoassay (Sharp et al. 1987). Samples were run in a single assay, in duplicate when sample volume permitted (>90% of all samples), diluted 1 in 2.3 in radioimmunoassay (RIA) buffer. Assay sensitivity was determined as described above, with a lower limit of 0.087$ng \cdot mL^{-1}$, after correcting for dilution. Samples with concentrations below this value were not included in analyses unless stated otherwise. The intra-assay coefficient of variation for this assay was 6.4% for a high value pool and 8.1% for a low value pool, and a curve generated by serial dilution of zebra finch plasma ran parallel to the standard curve within the range assayed.

2.2.3. Bromocriptine treatment

We used the dopamine (D2 and D3) receptor agonist, bromocriptine (2-bromo-α-ergocriptine mesylate; Enzo, PA, USA) to manipulate plasma PRL levels. Bromocriptine binds to the inhibitory D2 receptor on secretory lactotroph cells in the pituitary, and has been widely used to lower PRL in mammals and, less commonly, in birds (see references below). Females were randomly assigned to either one of two doses of

bromocriptine (low, 333µg/kg body weight or high, 3333µg/kg body weight w/v in DMSO (dimethylsulfoxide; Sigma-Aldrich, MO, USA), or vehicle only control. Doses were based on previous work in mammals (Bridges and Ronsheim 1990; Roberts et al. 2001; Bales et al. 2002) and birds (Jouventin and Mauget 1996; Angelier et al. 2006). Bromocriptine was administered by intra-muscular injection into the pectoral muscle, daily between 1100 and 1300 hours beginning the day the first egg was laid and terminating at clutch completion (described above). On egg day three of the bromocriptine experiment, injections were carried out immediately after blood sampling.

2.2.4. Data analyses

Data were first examined for normality, outliers, collinearity and interactions between explanatory variables. Both hormones showed deviations from normality, which was improved with log transformation. Log transformed data are described using median and interquartile range, otherwise data are stated as mean ± standard error. Repeatability was calculated using previously described methods (Lessells and Boag 1987). Since there were no statistical differences in the results found using mass alone or the residuals of a regression of mass by tarsus, mass alone was used as the measure of condition in all relevant analyses. Our breeding females exhibited a range of clutch sizes, including clutches outside the range observed in the wild (2-7 eggs; Zann 1996). We therefore considered clutches smaller than 2 eggs or larger than 7 'atypical' under normal breeding conditions and ran all analyses with and without these clutch sizes. For the bromocriptine experiment, we predicted individual increases in clutch size in response to the treatment, specifically those greater than the range observed in the wild, and so clutches > 7 eggs clutch sizes were considered 'normal' in these analyses.

Pre-breeding and simple breeding comparisons (excluding clutch size; see below) were conducted using ANOVA or ordinary least squares regression. To examine females through treatment and time (i.e. between pre-breeding and breeding; between control breeding and bromocriptine breeding), we used linear mixed effects models for repeated measures with individual female as a random factor, carried out in the statistical package 'nlme' in R 2.12.2 (R Core Development Team 2011; Pinheiro et al. 2011). This experimental and statistical design allowed us to make intra-individual comparisons of the effects of treatment, so that treated females were compared to

themselves under the untreated breeding conditions (in addition to retaining a vehicle only control group for bromocriptine, see above). Starting sample sizes for each analysis were: pre-breeding (78); untreated breeding (44); bromocriptine breeding (38). Thirty-three females sampled during the pre-breeding stage were reserved for other experiments (Ryan et al. in prep.), and were included in analyses only where applicable. For each stage, a small subset of females did not provide sufficient plasma for both hormone assays, had hormone values below the detection limit (described above), failed to breed, or laid less than 3 eggs (i.e. no hormone values for egg day three). As a result, model degrees of freedom vary, but are always based on the maximum number of available data points.

Since clutch size is a discrete count variable, all analyses of this trait were conducted using generalized linear or generalized linear mixed effects models, with quasipoisson family to account for underdispersion (R package "glmmPQL"; Fox and Weisberg 2011). Analyses of egg mass was conducted on all eggs within a clutch, with individual female as a random factor to account for pseudo-replication. All analyses were followed with standard model validation procedures to test the assumptions of the test employed. Data points with high leverage and Cook's distance (greater than 4/n) were considered influential, and outputs are presented for models including and excluding these points for transparency. Where multiple explanatory variables were found to affect a dependant variable, p-values are given for the full model including all significant variables (ANCOVA).

2.3. Results

2.3.1. Relationships between pre-breeding PRL, LH and measures of body condition

Mean female body mass and hematocrit (i.e. measures of body condition) were 14.9 ± 0.2g and 53.9 ± 0.5% at the time of pre-breeding blood sampling. Median pre-breeding PRL was 21.0 ng/mL (14.3 to 35.5 ng/mL), and pre-breeding luteinizing hormone (LH) was 0.27 ng/mL (0.17 to 0.41 ng/mL). There were no significant relationships between pre-breeding mass or hematocrit and LH ($F_{1,59}$ = 0.066, P = 0.798

and $F_{1,59}$ = 1.125, *P* = 0.293, respectively), or pre-breeding mass and pre-breeding PRL ($F_{1,72}$ = 0.590, *P* = 0.445). Pre-breeding PRL was significantly and positively correlated with both pre-breeding LH (Fig. 2.1) and hematocrit ($F_{2,54}$ = 7.10, *P* = 0.010 and $F_{2,54}$ = 4.90, *P* = 0.031, respectively).

2.3.2. Relationships between pre-breeding and breeding PRL and LH, and pre-breeding hormones and reproductive traits

Forty-four of the 78 females sampled during pre-breeding were paired and nearly all females (42/44) laid at least one egg. Compared to pre-breeding levels (above), LH was significantly higher at the 3-egg stage in breeding females (0.49 ng/mL, 0.28-0.67 ng/mL; $F_{1,28}$ = 11.19, *P* = 0.024). LH between the pre-breeding and breeding stages was repeatable (R = 0.524; 95% CI = 0.27, 0.778; *P* = 0.001). Pre-breeding LH was also negatively correlated with laying interval, i.e. females with higher pre-breeding LH had shorter intervals between pairing and laying of the first egg ($F_{2,24}$ = 6.67, *P* = 0.003; Fig. 2.2; controlling for the time elapsed between pre-breeding blood sampling and subsequent pairing). In contrast, pre-breeding LH was not significantly correlated with either clutch size or egg mass of the subsequent breeding attempt (*P* > 0.25 for both).

Breeding PRL levels at the 3-egg stage were markedly and significantly ($F_{1,37}$ = 667.7, *P* < 0.001) higher than pre-breeding levels (above), reaching a median of 201.6 ng/mL (184.6-221.2 ng/mL). Individual PRL levels between pre-breeding and breeding stages were not repeatable (*P* > 0.90). Clutch-size (Fig. 2.3), egg mass, and log laying interval were also all independent of pre-breeding PRL (P > 0.13 in all cases).

2.3.3. Relationships between breeding PRL, LH, and reproductive traits

Neither clutch size or egg mass were significantly correlated with plasma LH on egg day three for either the normal range of clutch sizes (*P* > 0.30 for both) or all observed clutch sizes (*P* > 0.28 for both). Egg mass was significantly correlated with both laying order (*P* = 0.016) and mass at pairing (*P* = 0.038) for the normal range of clutch sizes, while clutch size was independent of mass at pairing for both normal and all observed sized clutches (*P* > 0.40 for both).

In an analysis of data for all observed clutch sizes, variation in plasma PRL at the 3-egg stage was significantly related to variation in clutch size ($P < 0.001$; Fig. 2.4) and egg mass ($P < 0.012$). However, standard model validation procedures confirmed very high leverage and influence (Cook's distance) of two of these data points (clutch sizes = 10) on model outcome and accuracy. Including these two points entirely reversed the (non-significant) direction of association between PRL and either clutch size or egg mass observed when these two data points were deleted. Furthermore, the relationship between PRL and clutch size from the model including all observed clutch sizes was inconsistent with a linear negative relationship that we would predict for these traits based on the PRL-based mechanistic model, i.e. two females with ten egg clutches had significantly lower PRL than females laying smaller clutch sizes, but the one female laying a twelve egg clutch had *higher* PRL (Fig. 2.4). Analyses within the normal range of clutch sizes for this species satisfied all model assumptions, and showed no evidence of highly influential observations. Using these data, both clutch size and egg mass (controlling for laying order) were independent of breeding PRL on egg day three ($P > 0.75$ for both; Fig. 2.4) and laying interval ($P > 0.20$ for both).

2.3.4. PRL, LH and reproductive traits for bromocriptine experimental breeding

Of the 38 females paired for the bromocriptine experiment, we had prior breeding data for 36 females. Two females did not reach the three-egg stage, leaving us with 34 females available for comparisons between breeding attempts. LH and hematocrit both decreased significantly ($F_{2,17} = 11.75$ and $F_{2,29} = 25.65$, respectively, $P < 0.01$ for both) in individual females between the first, control and second, experimental breeding attempt. However, these effects were independent of treatment ($P > 0.15$), and changes in both traits were independent of changes in PRL ($P > 0.25$), clutch size ($P > 0.25$), or egg mass ($P > 0.85$) between the two breeding attempts. Contrary to our expectations, there were no significant differences in plasma PRL in individual females sampled during their bromocriptine-treatment breeding attempt when compared to their control breeding attempt ($F_{1,29} = 0.05$, $P = 0.821$). In fact, PRL between the control and bromocriptine breeding were repeatable (R = 0.49; 95% CI = 0.222-0.757; $P = 0.002$). Furthermore, PRL levels during the experimental breeding attempt were independent of treatment

($F_{2,31}$ = 1.44, P = 0.531). Likewise, clutch size did not differ significantly in individual females between the first, control and second, experimental breeding attempt ($F_{1,30}$ = 3.99, P = 0.055), and there were no effects of bromocriptine treatment on the change in clutch size between the two breeding attempts ($F_{2,30}$ = 2.40, P = 0.108).

2.3.5. Changes in PRL, LH, and clutch size between control and experimental breeding attempts

Since there was no effect of bromocriptine treatment we pooled all treatment groups for the experimental breeding attempt. Again, clutch size was independent of plasma PRL for all clutches as well as for those within the normal range only (P > 0.49 for both; Fig. 2.5). However, individual *changes* in PRL levels between a females first, control and second, experimental breeding attempts were significantly negatively correlated with individual changes in clutch size, including (P = 0.031) or excluding two influential data points with only a single observation for a given change in clutch size (P = 0.041; Fig. 2.6).

2.4. Discussion

The objectives of this study were to determine: 1) the relationship between measures of condition (i.e. mass, hematocrit) and plasma PRL and LH for pre-breeding and breeding individual female zebra finches; 2) the relationship between pre-breeding hormones and subsequent clutch size, and; 3) the relationship between plasma PRL and LH and clutch size at the putative time of clutch size determination, for both control and experimental breeding attempts. We found no effect of pre-breeding PRL or LH on subsequent clutch size, however pre-breeding LH was negatively correlated with the time between pairing and the onset of laying. We also found no evidence for an inverse relationship between plasma PRL and plasma LH levels which would have been consistent with an inhibitory effect of PRL on LH. In contrast to previous studies (Badyaev and Duckworth 2005; Reddy et al. 2007) we observed no effect of bromocriptine on PRL. Nonetheless, and most importantly, we found no evidence to support a causal relationship between breeding plasma PRL levels and clutch size in two separate breeding attempts (the control breeding and the experimental,

bromocriptine breeding). Naturally occurring individual changes in plasma PRL between one breeding attempt and the next were however associated with individual changes in clutch size. These latter data support individually variable thresholds for an inhibitory effect of PRL on clutch size determination, though not via inhibitory effects on plasma LH.

2.4.1. Relationships between pre-breeding PRL, LH and measures of body condition

We first examined pre-breeding PRL and LH and condition-related traits (e.g. body mass, hematocrit) to test the hypothesis that individual variability in these characteristics could be predictive of subsequent reproductive performance (Chastel et al. 2003; Ouyang et al. 2011; Crossin et al. 2012). We did find a positive and significant relationship between pre-breeding hematocrit and PRL, however we observed no relationship between pre-breeding body mass and pre-breeding PRL or LH, nor any relationship between pre-breeding hematocrit and LH. We also found no effect of pre-breeding mass, hematocrit, PRL or LH on subsequent clutch size. These results do not support the hypothesis that plasma PRL or LH prior to breeding provide an early 'window' into subsequent reproductive performance, at least in the form of clutch size (but see "reproductive readiness", below).

Plasma PRL and LH were significantly, positively correlated in pre-breeding female zebra finches. This contrasts with results from some studies, mostly in breeding poultry, demonstrating an inhibitory effect of PRL on LH hormone titres or LH mRNA expression (Rozenboim et al. 1993; You et al. 1995). However, our results in pre-breeding females, where PRL levels are relatively low, are consistent with growing evidence that PRL can have both inhibitory *and* stimulatory effects on gonadal function, depending on reproductive state and PRL concentration (Maney et al. 1999; Hrabia et al. 2004; Small et al. 2007; Li et al. 2011). The origin of the positive correlation between PRL and LH is not obvious; LH activates the reproductive axis and steroidogenesis, and steroid hormones can stimulate PRL secretion (El Halawani et al. 1983; Mauro et al. 1992). However, since non-photoperiodic cues (e.g. social stimuli) likely contribute to variation in pre-breeding LH levels in opportunistically breeding species like the zebra finch (e.g. Maney et al. 1999; Small et al. 2007; Perfito et al. 2007), pre-breeding LH and

PRL may reflect individual differences in the relative activation of the reproductive axis prior to actual onset of egg-laying, i.e. individual 'reproductive readiness'.

2.4.2. Relationships between pre-breeding PRL, LH, and reproductive traits

Individual differences in reproductive readiness are supported in our study by the link between pre-breeding LH levels and the interval between pairing and laying. Females with relatively high pre-breeding LH were the quickest to initiate laying. Presumably, variability in pre-breeding LH is indicative of the differences in the developmental state of the ovary and nascent follicles, a suggestion supported by other work in captive pre-breeding zebra finches (see Fig. 4 in Perfito 2010). The finding that not all females are in a homogeneous pre-breeding state is of critical importance to laboratory studies of reproductive behaviour, particularly those involving the timing of breeding or response to mating stimuli (Perfito 2010). In contrast to LH, pre-breeding PRL was not predictive of the interval between pairing and laying, contrary to previous work in free-living House Sparrows (*Passer domesticus*), in which females with high PRL prior to breeding laid their first egg sooner (Ouyang et al. 2011). However, as in our study, Schaper et al. (2012) also failed to detect any relationship between pre-breeding PRL and readiness to lay under controlled laboratory conditions, suggesting an independent role for photoperiod on PRL and activation of the reproductive-axis, possibly via independent control of PRL and LH secretion.

A key component of the PRL-based model for clutch size determination is that PRL exerts anti-gonadal effects indirectly via the inhibition of LH expression at the level of the pituitary (Lea et al. 1981; Sockman et al. 2006). This component of the model predicts an inverse relationship between these hormones, at least at the time of clutch size determination. We were able to examine the relationship between these two hormones, and how they changed over time, by tracking individual hormonal profiles through the transition between pre-breeding and breeding states. Breeding LH levels were moderately though significantly higher than pre-breeding levels, and were repeatable between pre-breeding and breeding LH. In contrast, PRL rose dramatically (as high as 27 fold) between pre-breeding and egg day 3, and PRL levels on egg day 3 were independent of pre-breeding PRL. Although LH levels on day three were probably

beginning to decline (based on rapid decreases in estradiol around this time; Williams et al. 2005), our data still suggest an uncoupling of the positive correlation between PRL and LH that we observed in pre-breeding females. An uncoupling of these two hormones over time does not support the idea of an systemic inhibitory effect of PRL on LH, since in our study both hormones increase with breeding, yet vary independently between pre-breeding and breeding states. Accordingly, we also found no significant relationship between breeding levels of PRL and LH. Furthermore, while experimental bromocriptine treatment had no effect on circulating PRL (discussed below), we also found no evidence for an inhibitory effect of PRL on LH in our experimental breeding. Though correlational, the lack of empirical support for an inhibitory effect of PRL on LH in this study, as well as in other passerines (Meier and Dusseau 1968; Buntin et al. 1999; Small et al. 2007), raises questions about the universality of the PRL-dependant control of LH in the current mechanistic hypothesis, and its applicability in this taxon.

2.4.3. Effect of bromocriptine on PRL levels

In contrast to previous studies on mammals (Palestine et al. 1987; Bridges and Ronsheim 1990) and birds (Jouventin and Mauget 1996; Angelier et al. 2006; Reddy et al. 2007) we found that bromocriptine treatment had no effect on circulating PRL levels in zebra finches for either the low or high dose groups, nor did we observe a treatment effect on clutch size between the control and experimental breeding. While a range of bromocriptine doses have been employed in birds, from as low as 14 $\mu g \cdot kg^{-1} \cdot day^{-1}$ (Reddy et al. 2007) to as high as 10,000 $\mu g \cdot kg^{-1} \cdot day^{-1}$ (Badyaev and Duckworth 2005), our doses (low: 333 $\mu g \cdot kg^{-1} \cdot day^{-1}$; high: 3,333 $\mu g \cdot kg^{-1} \cdot day^{-1}$) are comparable to those successfully employed in other avian studies (Jouventin and Mauget 1996: 4,167 $\mu g \cdot kg^{-1} \cdot day^{-1}$; Angelier et al. 2006: 1,500 $\mu g \cdot kg^{-1} \cdot day^{-1}$) and commonly used in mammals (Palestine et al. 1987: 1,800 $\mu g \cdot kg^{-1} \cdot day^{-1}$; Bridges and Ronsheim 1990: 4,000 $\mu g \cdot kg^{-1} \cdot day^{-1}$). The reason for the failure of bromocriptine to effect PRL levels in our study is not clear, but is not restricted to *T. guttata* (e.g. bromocriptine had no effect on PRL in *Rissa tridactyla*; F. Angelier, pers. comm.). Nonetheless, several factors could explain differences in responsivity to bromocriptine among different avian species. PRL secretion from avian lactotrophs is predominantly under the stimulatory control of vasointestinal peptide (VIP; Sharp et al. 1998), with inhibitory type-2 dopamine (D2)

receptors (the target for bromocriptine) only identified on PRL-secreting lactotrophs in turkeys (Al Kahtane et al. 2003). Thus, the extent to which dopamine (DA) inhibits avian PRL secretion more generally is unknown. Indeed, intra-specific polymorphisms in the promoter region of the PRL gene suggest variability in the regulatory control of PRL gene expression (Zadworny et al. 2002; Cui et al. 2006; Hiyama et al. 2009b). Polymorphisms in the D2 receptor itself have been linked to variation in incubation behaviour, and could further alter the inhibitory capacity of the D2 receptor on PRL gene expression (Chaiseha et al. 2003; Xu et al. 2010). Inter-specific differences in VIP gene sequence with the capacity to affect the regulatory control of PRL have also been described (Hiyama et al. 2009a). Cumulatively, this genetic variability could underlie individual differences in the inhibitory role of the D2 receptor in PRL secretion, and could explain intra- or inter-specific variability in the efficacy of bromocriptine at suppressing PRL secretion in birds.

2.4.4. PRL, LH and reproductive traits for control and bromocriptine breeding attempts

The PRL-based mechanism for clutch size determination predicts a clear negative relationship between these two traits, i.e. females with higher circulating PRL early during laying should lay smaller clutches, due to the earlier and/or greater inhibitory effect of elevated plasma PRL (Sockman et al. 2000). We found that breeding PRL levels during what is believed to be a critical period in the clutch size determination in the zebra finch were not associated with differences in clutch size. These data suggest that absolute circulating PRL levels at the time when follicular inhibition is thought to occur in this species (Haywood 1993) are not by themselves sufficient to explain variation in clutch size. This conclusion is supported by the results of our bromocriptine experiment. Although there were no treatment effects of bromocriptine on PRL, results for this breeding mirrored those of the unmanipulated breeding; there was no significant relationship between experimental breeding PRL and clutch size. In the experimental (i.e. bromocriptine) breeding, the one female who laid an atypically large clutch (10 eggs, for the second time) displayed PRL levels that were no different from females laying other clutch sizes, again supporting the conclusion that absolute PRL levels themselves do not play a role in determining clutch size in this species.

2.4.5. Potential sources of individual variability in PRL-based regulation of clutch size

The current model for clutch size determination has focused on an inhibitory role for circulating plasma PRL early on during laying (Haywood 1993; Sockman et al. 2000), yet our results suggest that individual variation in absolute plasma PRL is not involved clutch size determination. We found no relationship between clutch size and pre-breeding or breeding PRL, nor any evidence for an inhibitory effect of PRL on LH. While we observed no effect of bromocriptine on PRL, the lack of support for the mechanistic model observed in our first study was corroborated in the experimental breeding attempt. Given our sample sizes and the range of clutch sizes, as well as the tightly controlled diet, photoperiod, age and reproductive history of the individuals included in the study, we believe our study provides a robust test of the PRL-based model for clutch size determination, whereby *circulating* PRL provides the regulatory control. Alternative mechanisms, still involving PRL, are still worth considering. Absolute hormone levels taken at a single time point may not adequately reflect the complexity of endocrine networks (Breuner and Orchinik 2002; Aderem 2005; Hau et al. 2011). Differential PRL receptor expression among individuals, polymorphisms in gene and receptor, tissue specific-receptor expression, binding proteins, and post-translational modifications (e.g. glycosylation and phosphorylation) could all affect the biological activity and effects of PRL at a given plasma concentration (Zadworny et al. 2002). Several of these sources of variability have been described in the PRL gene itself (Kansaku et al. 2008; Jiang et al. 2009; Hiyama et al. 2009b; Bhattacharya et al. 2011), its receptor (Ohkubo et al. 1998; Leclerc et al. 2007), or its regulators (Chaiseha et al. 2003; Hiyama et al. 2009a; Xu et al. 2010). As yet, few studies in avian endocrinology, and none examining avian clutch size determination, account for these potential complexities in endocrinological systems (Aderem 2005). Yet we expect many differences, such as gene and receptor polymorphisms, to show greater variation between rather than within individuals.

2.4.6. Individual changes in PRL, LH, and clutch size between control and experimental breeding attempts

While the PRL-based mechanism for clutch size determination does not appear to involve a constant threshold at the scale of the population (i.e. that some absolute

PRL level terminates laying), individual differences in either the rate of increase (slope) or in the inhibitory threshold (relative PRL level for inhibition for a given breeding attempt) remain plausible alternatives to, or modifications of, the mechanistic model in its current form (Meijer et al. 1990; Williams 2012b, p. 186). Using data from our two breeding events, we were able to examine changes in PRL relative to changes in clutch size, giving us insights into individual co-variability in these two traits. We observed a significant association between changes in PRL and changes in clutch size. Females whose PRL levels rose from one breeding event to the next were likely to decrease the size of clutch they laid, in contrast to females whose PRL levels fell (who increased the size of clutch they laid). Since the changes in PRL between breeding attempts were not associated with changes in LH, nor were changes in LH associated changes in clutch size, neither PRL nor clutch size appear to be related through the upper levels of the HPG-axis. The broader implications of these results are not clear, but may imply downstream regulatory effects of PRL (e.g. at the level of the ovary). Although speculative, this hypothesis is supported by work demonstrating the presence of PRL receptors in ovarian follicles (Ohkubo et al. 1998), which can directly inhibit the effects of follicle-stimulating hormone (FSH) and LH on, as well as estrogen and progesterone secretion from, the avian ovary (Li and Yang 1995; Hrabia et al. 2004).

2.4.7. Conclusions

In conclusion, studying avian clutch size determination by looking at individual co-variation in PRL and egg number may suggest more biologically-relevant alternatives to the mechanistic hypothesis in its current form, a hypothesis we found no support for in this study. Further experimental work successfully uncoupling PRL from clutch size would help reinforce this conclusion. If the hormonal regulatory control of clutch size is superimposed upon individual variation in downstream effectors (e.g. receptor expression in the ovary), repeated measurements of individuals through time, as conducted in this study, have the benefit of eliminating at least a portion these potentially confounding effects.

2.5. References

Aderem, Alan. 2005. “Systems Biology: Its Practice and Challenges.” *Cell* 121 (4) (May 20): 511–513. doi:10.1016/j.cell.2005.04.020.

Al Kahtane, A, Y Chaiseha, and M El Halawani. 2003. “Dopaminergic regulation of avian prolactin gene transcription.” *Journal of Molecular Endocrinology* 31 (1): 185 – 196. doi:10.1677/jme.0.0310185.

Angelier, Frédéric, Christophe Barbraud, Hervé Lormée, François Prud’homme, and Olivier Chastel. 2006. “Kidnapping of chicks in emperor penguins: a hormonal by-product?” *Journal of Experimental Biology* 209 (8) (April 15): 1413 –1420. doi:10.1242/jeb.02138.

Angelier, Frederic, and Olivier Chastel. 2009. “Stress, prolactin and parental investment in birds: A review.” *General and comparative endocrinology* 163 (1-2) (September): 142–148.

Badyaev, Alex V., and Renée A. Duckworth. 2005. Evolution of plasticity in hormonally-mediated parental tactics: An example in the house finch. In *Functional Avian Endocrinology*, ed. by Alistair Dawson and Peter J. Sharp. Alpha Science Int’l Ltd.

Bailey, Robert E. 1950. “Inhibition with Prolactin of Light-Induced Gonad Increase in White-Crowned Sparrows.” *The Condor* 52 (6) (November 1): 247–251. doi:10.2307/1364516.

Bales, Karen, Jeffrey A. French, and James M. Dietz. 2002. “Explaining variation in maternal care in a cooperatively breeding mammal.” *Animal Behaviour* 63 (3) (March): 453–461. doi:10.1006/anbe.2001.1954.

Bentley, G.E., A.R. Goldsmith, A. Dawson, L.M. Glennie, R.T. Talbot, and P.J. Sharp. 1997. “Photorefractoriness in European Starlings (*Sturnus vulgaris*) Is Not Dependent upon the Long-Day-Induced Rise in Plasma Thyroxine.” *General and Comparative Endocrinology* 107 (3) (September): 428–438. doi:10.1006/gcen.1997.6941.

Bhattacharya, T.K., R.N. Chatterjee, R.P. Sharma, M. Niranjan, and U. Rajkumar. 2011. “Associations between novel polymorphisms at the 5’-UTR region of the prolactin gene and egg production and quality in chickens.” *Theriogenology* 75 (4) (March 1): 655–661. doi:16/j.theriogenology.2010.10.005.

Breuner, C. W., and M. Orchinik. 2002. “Plasma binding proteins as mediators of corticosteroid action in vertebrates.” *Journal of Endocrinology* 175 (1) (October 1): 99–112. doi:10.1677/joe.0.1750099.

Bridges, Robert S., and Paul M. Ronsheim. 1990. “Prolactin (PRL) Regulation of Maternal Behaviour in Rats: Bromocriptine Treatment Delays and PRL Promotes the Rapid Onset of Behaviour.” *Endocrinology* 126 (2) (February 1): 837 –848. doi:10.1210/endo-126-2-837.

Buntin, J.D., J.P. Advis, M.A. Ottinger, R.W. Lea, and P.J. Sharp. 1999. “An Analysis of Physiological Mechanisms Underlying the Antigonadotropic Action of Intracranial Prolactin in Ring Doves.” *General and Comparative Endocrinology* 114 (1) (April): 97–107. doi:10.1006/gcen.1998.7239.

Chaiseha, Yupaporn, Orlan Youngren, Khaled Al-Zailaie, and Mohamed El Halawani. 2003. “Expression of D_1 and D_2 Dopamine Receptors in the Hypothalamus and Pituitary during the Turkey Reproductive Cycle: Colocalization with Vasoactive Intestinal Peptide.” *Neuroendocrinology* 77 (2): 105–118. doi:10.1159/000068649.

Charmantier, Anne, Christopher Perrins, Robin H. McCleery, and Ben C. Sheldon. 2006. “Evolutionary Response to Selection on Clutch Size in a Long‐Term Study of the Mute Swan.” *The American Naturalist* 167 (3) (March 1): 453–465.

Chastel, Olivier, André Lacroix, and Marcel Kersten. 2003. “Pre-breeding energy requirements: thyroid hormone, metabolism and the timing of reproduction in house sparrows *Passer domesticus*.” *Journal of Avian Biology* 34 (3): 298–306. doi:10.1034/j.1600-048X.2003.02528.x.

Crossin, Glenn T., Richard A. Phillips, Phil N. Trathan, Derren S. Fox, Alistair Dawson, Katherine E. Wynne-Edwards, and Tony D. Williams. 2012. “Migratory carryover effects and endocrinological correlates of reproductive decisions and reproductive success in female albatrosses.” *General and Comparative Endocrinology* 176 (2) (April 1): 151–157. doi:10.1016/j.ygcen.2012.01.006.

Cui, J. X., H. L. Du, Y. Liang, X. M. Deng, N. Li, and X. Q. Zhang. 2006. “Association of Polymorphisms in the Promoter Region of Chicken Prolactin with Egg Production.” *Poultry Science* 85 (1) (January 1): 26–31.

Decker, Karie, Courtney Conway, and Joseph Fontaine. 2012. “Nest predation, food, and female age explain seasonal declines in clutch size.” *Evolutionary Ecology* 26 (3): 683–699. doi:10.1007/s10682-011-9521-7.

El Halawani, M. E., W. H. Burke, J. R. Millam, S. C. Fehrer, and B. M. Hargis. 1984. “Regulation of prolactin and its role in gallinaceous bird reproduction.” *Journal of Experimental Zoology* 232 (3) (December): 521–529. doi:10.1002/jez.1402320319.

El Halawani, M.E., J.L. Silsby, S.C. Fehrer, and E.J. Behnke. 1983. “Effects of estrogen and progesterone on serum prolactin and luteinizing hormone levels in ovariectomized turkeys (*Meleagris gallopavo*).” *General and Comparative Endocrinology* 52 (1) (October): 67–78. doi:10.1016/0016-6480(83)90159-4.

Flint, Paul L., J. Barry Grand, Thomas F. Fondell, and Julie A. Morse. 2006. “Population Dynamics of Greater Scaup Breeding on the Yukon-Kuskokwim Delta, Alaska.” *Wildlife Monographs* 162 (1): 1–22. doi:10.2193/0084-0173(2006)162[1:PDOGSB]2.0.CO;2.

Fox, J., and Sanford Weisberg. 2011. *An R companion to applied Regression*. Second. Thousand Oaks CA: Sage. http://socserv.socsci.mcmaster.ca/jfox/Books/Companion.

Godfray, H. C. J., L. Partridge, and P. H. Harvey. 1991. “Clutch Size.” *Annual Review of Ecology and Systematics* 22 (January 1): 409–429.

Greives, Timothy J., Sjouke A. Kingma, Giulia Beltrami, and Michaela Hau. 2012. “Melatonin delays clutch initiation in a wild songbird.” *Biology Letters* 8 (3) (June 23): 330–332. doi:10.1098/rsbl.2011.1100.

Haftorn, Svein. 1981. “Incubation during the Egg-Laying Period in Relation to Clutch-Size and Other Aspects of Reproduction in the Great Tit *Parus major*.” *Ornis Scandinavica* 12 (3) (December 1): 169–185. doi:10.2307/3676074.

Hau, M, JC Wingfield, T Flatt, and A Heyland. 2011. “Hormonally-regulated trade-offs: evolutionary variability and phenotypic plasticity in testosterone signaling pathways.” *Mechanisms of Life History Evolution (T. Flatt and A. Heyland, Eds.). Oxford University Press, New York*: 349–361.

Haywood, Sacha. 1993. “Sensory Control of Clutch Size in the Zebra Finch (*Taeniopygia guttata*).” *The Auk* 110 (4) (October 1): 778–786.

Hiyama, Gen, Tsukasa Sato, David Zadworny, and Norio Kansaku. 2009a. “Cloning of PRL and VIP cDNAs of the Java sparrow (*Padda oryzivora*).” *Animal Science Journal* 80 (2) (April): 176–186.

Hiyama, Gen, David Zadworny, and Norio Kansaku. 2009b. “Cloning and Comparison of Prolactin Promoter in Galliformes.” *The Journal of Poultry Science* 46 (1): 6–12.

Hrabia, A., H. Paczoska-Eliasiewicz, and J. Rzasa. 2004. “Effect of prolactin on estradiol and progesterone secretion by isolated chicken ovarian follicles.” *Folia Biologica, 52* 3 (4): 197–203.

Jiang, R. S., L. L. Zhang, Z. Y. Geng, T. Yang, and S. S. Zhang. 2009. “Single nucleotide polymorphisms in the 5’-flanking region of the prolactin gene and the association with reproduction traits in geese (Short communication).” *South African Journal of Animal Science* 39 (1). http://www.ajol.info/index.php/sajas/article/view/43550.

Jouventin, P., and R. Mauget. 1996. “The endocrine basis of the reproductive cycle in the king penguin (*Aptenodytes patagonicus*).” *Journal of Zoology* 238 (4): 665–678. doi:10.1111/j.1469-7998.1996.tb05421.x.

Kansaku, Norio, Gen Hiyama, Tomohiro Sasanami, and David Zadworny. 2008. "Prolactin and Growth Hormone in Birds: Protein Structure, Gene Structure and Genetic Variation." *The Journal of Poultry Science* 45 (1): 1–6.

Klomp, H. 1970. "The determination of clutch-size in birds: a review." *Ardea*.

Lack, David. 1947. "The Significance of Clutch-size." *Ibis* 89 (2): 302–352. doi:10.1111/j.1474-919X.1947.tb04155.x.

Lea, R. W., A. S. M. Dods, P. J. Sharp, and A. Chadwick. 1981. "The Possible Role of Prolactin in the Regulation of Nesting Behaviour and the Secretion of Luteinizing Hormone in Broody Bantams." *Journal of Endocrinology* 91 (1) (October 1): 89–97. doi:10.1677/joe.0.0910089.

Leclerc, B., D. Zadworny, G. Bedecarrats, and U. Kuhnlein. 2007. "Development of a real-time (Q) PCR assay to measure variation in expression of prolactin receptor mRNA in the hypothalamus and pituitary gland during late embryogenesis in turkeys and chickens." *General and comparative endocrinology* 150 (2) (January 15): 319–325.

Lessells, C. M., and Peter T. Boag. 1987. "Unrepeatable Repeatabilities: A Common Mistake." *The Auk* 104 (1) (January 1): 116–121. doi:10.2307/4087240.

Lessells, C. M., F. Cooke, and R. F. Rockwell. 1989. "Is there a trade-off between egg weight and clutch size in wild Lesser Snow Geese (*Anser c. caerulescens*)?" *Journal of Evolutionary Biology* 2 (6): 457–472.

Li, W.L., Y. Liu, Y.C. Yu, Y.M. Huang, S.D. Liang, and Z.D. Shi. 2011. "Prolactin plays a stimulatory role in ovarian follicular development and egg laying in chicken hens." *Domestic Animal Endocrinology* 41 (2) (August): 57–66. doi:16/j.domaniend.2011.03.002.

Li, Y.H., and C.R. Yang. 1995. "Effect of prolactin on the steroidogenesis and proliferation of cultured theca cells from domestic hens." *Poult. Avian Biol. Rev. 6: Abstract* 295.

Maney, Donna L., Thomas P. Hahn, Stephan J. Schoech, Peter J. Sharp, Martin L. Morton, and John C. Wingfield. 1999. "Effects of Ambient Temperature on Photo-Induced Prolactin Secretion in Three Subspecies of White-Crowned Sparrow, *Zonotrichia leucophrys*." *General and Comparative Endocrinology* 113 (3) (March): 445–456. doi:10.1006/gcen.1998.7219.

Massaro, Melanie, Alvin N. Setiawan, and Lloyd S. Davis. 2007. "Effects of artificial eggs on prolactin secretion, steroid levels, brood patch development, incubation onset and clutch size in the yellow-eyed penguin (*Megadyptes antipodes*)." *General and Comparative Endocrinology* 151 (2) (April): 220–229. doi:10.1016/j.ygcen.2007.01.034.

Mauro, Laura J., Orlan M. Youngren, John A. Proudman, Richard E. Phillips, and Mohamed E. El Halawani. 1992. “Effects of reproductive status, ovariectomy, and photoperiod on vasoactive intestinal peptide in the female turkey hypothalamus.” *General and Comparative Endocrinology* 87 (3) (September): 481–493. doi:10.1016/0016-6480(92)90056-P.

McCleery, R.H., R.A. Pettifor, P. Armbruster, K. Meyer, B.C. Sheldon, and C. Perrins. 2004. “Components of Variance Underlying Fitness in a Natural Population of the Great Tit Parus major.” *The American Naturalist* 164 (3) (September 1): E62–E72.

Meier, Albert H. 1969. “Antigonadal effects of prolactin in the white-throated sparrow, Zonotrichia albicollis.” *General and Comparative Endocrinology* 13 (2) (October): 222–225. doi:10.1016/0016-6480(69)90243-3.

Meier, Albert H., and Jerry W. Dusseau. 1968. “Prolactin and the Photoperiodic Gonadal Response in Several Avian Species.” *Physiological Zoology* 41 (1) (January 1): 95–103. doi:10.2307/30158487.

Meijer, Theo, Serge Daan, and Michael Hall. 1990. “Family Planning in the Kestrel (*Falco tinnunculus*): The Proximate Control of Covariation of Laying Date and Clutch Size.” *Behaviour* 114 (1/4): 117–136.

Miller, David A., Carol M. Vleck, and David L. Otis. 2009. “Individual variation in baseline and stress-induced corticosterone and prolactin levels predicts parental effort by nesting mourning doves.” *Hormones and behaviour* 56 (4) (October): 457–464.

Murton, R.K., and N.J. Westwood. 1977. *Avian breeding cycles*. Oxford [etc.]: Clarendon Press.

Nager, R. G, P. Monaghan, and D. C Houston. 2000. “Within-clutch trade-offs between the number and quality of eggs: experimental manipulations in gulls.” *Ecology* 81 (5): 1339–1350.

O’Dwyer, T. W., W. A. Buttemer, D. M. Priddel, and J. A. Downing. 2006. “Prolactin, body condition and the cost of good parenting: an interyear study in a long-lived seabird, Gould’s Petrel (*Pterodroma leucoptera*).” *Functional Ecology* 20 (5): 806–811. doi:10.1111/j.1365-2435.2006.01168.x.

Ohkubo, T., M. Tanaka, K. Nakashima, R.T. Talbot, and P.J. Sharp. 1998. “Prolactin Receptor Gene Expression in the Brain and Peripheral Tissues in Broody and Nonbroody Breeds of Domestic Hen.” *General and Comparative Endocrinology* 109 (1) (January): 60–68. doi:10.1006/gcen.1997.7008.

Ouyang, Jenny Q., Peter J. Sharp, Alistair Dawson, Michael Quetting, and Michaela Hau. 2011. “Hormone levels predict individual differences in reproductive success in a passerine bird.” *Proceedings of the Royal Society B: Biological Sciences* (January 19). doi:10.1098/rspb.2010.2490. http://rspb.royalsocietypublishing.org/content/early/2011/01/11/rspb.2010.2490.abstract.

Palestine, A G, C G Muellenberg-Coulombre, M K Kim, M C Gelato, and R B Nussenblatt. 1987. “Bromocriptine and low dose cyclosporine in the treatment of experimental autoimmune uveitis in the rat.” *Journal of Clinical Investigation* 79 (4) (April): 1078–1081.

Perfito, Nicole. 2010. “The reproductive and stress physiology of Zebra Finches in context: integrating field and laboratory studies.” *Emu* 110 (3): 199–208.

Perfito, Nicole, Richard A. Zann, George E. Bentley, and Michaela Hau. 2007. “Opportunism at work: habitat predictability affects reproductive readiness in free-living zebra finches.” *Functional Ecology* 21 (2): 291–301. doi:10.1111/j.1365-2435.2006.01237.x.

Pettifor, R. A., C. M. Perrins, and R. H. McCleery. 1988. “Individual optimization of clutch size in great tits.” *Nature* 336 (6195) (November 10): 160–162. doi:10.1038/336160a0.

Pinheiro, J, D Bates, S DebRoy, D Sarkar, and the R Development Core Team. 2011. “nlme: Linear and nonlinear mixed effects models.” *R package version 3.1-98*.

R Core Development Team. 2011. “R: A language and environment for statistical computing, reference index version 2.12.2.” *R Foundation for Statistical Computing, Vienna, Austria.*

Reddy, I. J., C. G. David, and S. S. Raju. 2007. “Effect of suppression of plasma prolactin on luteinizing hormone concentration, intersequence pause days and egg production in domestic hen.” *Domestic animal endocrinology* 33 (2) (August): 167–175.

Ricklefs, R. E, and M. Wikelski. 2002. “The physiology/life-history nexus.” *Trends in Ecology & Evolution* 17 (10): 462–468.

Ricklefs, Robert E. 2010. “Parental Investment and Avian Reproductive Rate: Williams’s Principle Reconsidered.” *The American Naturalist* 175 (3) (March 1): 350–361.

Roberts, R L, K T Jenkins, T Lawler Jr, F H Wegner, and J D Newman. 2001. “Bromocriptine administration lowers serum prolactin and disrupts parental responsiveness in common marmosets (*Callithrix j. jacchus*).” *Hormones and Behaviour* 39 (2) (March): 106–112. doi:10.1006/hbeh.2000.1639.

Rockwell, R. F., C. S. Findlay, and F. Cooke. 1987. “Is there an Optimal Clutch Size in Snow Geese?” *The American Naturalist* 130 (6) (December 1): 839–863.

Rozenboim, I., C. Tabibzadeh, J. L. Silsby, and M. E. el Halawani. 1993. “Effect of ovine prolactin administration on hypothalamic vasoactive intestinal peptide (VIP), gonadotropin releasing hormone I and II content, and anterior pituitary VIP receptors in laying turkey hens.” *Biology of Reproduction* 48 (6) (June 1): 1246–1250. doi:10.1095/biolreprod48.6.1246.

Schaper, Sonja V., Alistair Dawson, Peter J. Sharp, Samuel P. Caro, and Marcel E. Visser. 2012. “Individual variation in avian reproductive physiology does not reliably predict variation in laying date.” *General and Comparative Endocrinology* 179 (1) (October 1): 53–62. doi:10.1016/j.ygcen.2012.07.021.

Sharp, Peter J., Alistair Dawson, and Robert W. Lea. 1998. “Control of luteinizing hormone and prolactin secretion in birds.” *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology* 119 (3) (June): 275–282. doi:10.1016/S0742-8413(98)00016-4.

Sharp, PJ, IC Dunn, and RT Talbot. 1987. “Sex-Differences in the LH Responses to Chicken LHRH-I and LHRH-II in the Domestic-Fowl.” *Journal of Endocrinology* 115 (2) (November): 323–331. doi:10.1677/joe.0.1150323.

Sibly, Richard M., Christopher C. Witt, Natalie A. Wright, Chris Venditti, Walter Jetz, and James H. Brown. 2012. “Energetics, lifestyle, and reproduction in birds.” *Proceedings of the National Academy of Sciences* 109 (27) (July 3): 10937–10941. doi:10.1073/pnas.1206512109.

Small, Thomas W., Peter J. Sharp, and Pierre Deviche. 2007. “Environmental regulation of the reproductive system in a flexibly breeding Sonoran Desert bird, the Rufous-winged Sparrow, *Aimophila carpalis*.” *Hormones and Behaviour* 51 (4) (April): 483–495. doi:10.1016/j.yhbeh.2007.01.004.

Sockman, K. W., H. Schwabl, and P. J. Sharp. 2000. “The role of prolactin in the regulation of clutch size and onset of incubation behaviour in the American kestrel.” *Hormones and behaviour* 38 (3) (November): 168–176.

Sockman, Keith W., Peter J. Sharp, and Hubert Schwabl. 2006. “Orchestration of avian reproductive effort: an integration of the ultimate and proximate bases for flexibility in clutch size, incubation behaviour, and yolk androgen deposition.” *Biological Reviews* 81 (04) (October): 629. doi:10.1017/S1464793106007147.

Travers, Marc, Michael Clinchy, Liana Zanette, Rudy Boonstra, and Tony D. Williams. 2010. “Indirect predator effects on clutch size and the cost of egg production.” *Ecology Letters* 13 (8): 980–988. doi:10.1111/j.1461-0248.2010.01488.x.

Williams, George C. 1966. “Natural Selection, the Costs of Reproduction, and a Refinement of Lack’s Principle.” *The American Naturalist* 100 (916) (November 1): 687–690.

Williams, Tony D. 2012a. “Hormones, life-history, and phenotypic variation: Opportunities in evolutionary avian endocrinology.” *General and Comparative Endocrinology* (0). doi:10.1016/j.ygcen.2011.11.028. http://www.sciencedirect.com/science/article/pii/S0016648011004424.

———. 2012b. *Physiological Adaptations for Breeding in Birds*. Princeton, NJ: Princeton University Press, August 5.

Williams, Tony D., Caroline E. Ames, Yiannis Kiparissis, and Katherine E. Wynne-Edwards. 2005. “Laying–sequence–specific variation in yolk oestrogen levels, and relationship to plasma oestrogen in female zebra finches (*Taeniopygia guttata*).” *Proceedings of the Royal Society B: Biological Sciences* 272 (1559) (January 22): 173 –177. doi:10.1098/rspb.2004.2935.

Xu, H. P., X. Shen, M. Zhou, C. L. Luo, L. Kang, Y. Liang, H. Zeng, Q. H. Nie, D. X. Zhang, and X. Q. Zhang. 2010. “The dopamine D2 receptor gene polymorphisms associated with chicken broodiness.” *Poultry Science* 89 (3) (March 1): 428–438. doi:10.3382/ps.2009-00428.

You, S., L. K. Foster, J. L. Silsby, M. E. El Halawani, and D. N. Foster. 1995. “Sequence analysis of the turkey LH β subunit and its regulation by gonadotrophin-releasing hormone and prolactin in cultured pituitary cells.” *Journal of Molecular Endocrinology* 14 (1) (February 1): 117–129. doi:10.1677/jme.0.0140117.

Zadworny, D, N Kansaku, G Bedecarrats, D Guemene, and U Kuhnlein. 2002. “Prolactin and its receptor in galliformes.” *Avian and Poultry Biology Reviews* 13 (3): 7. doi:10.3184/147020602783698566.

Zanette, Liana Y, Aija F White, Marek C Allen, and Michael Clinchy. 2011. “Perceived Predation Risk Reduces the Number of Offspring Songbirds Produce per Year.” *Science* 334 (6061) (December 9): 1398–1401. doi:10.1126/science.1210908.

Zann, R.A. 1996. *The zebra finch: a synthesis of field and laboratory studies*. Oxford University Press Oxford:

2.6. Figures

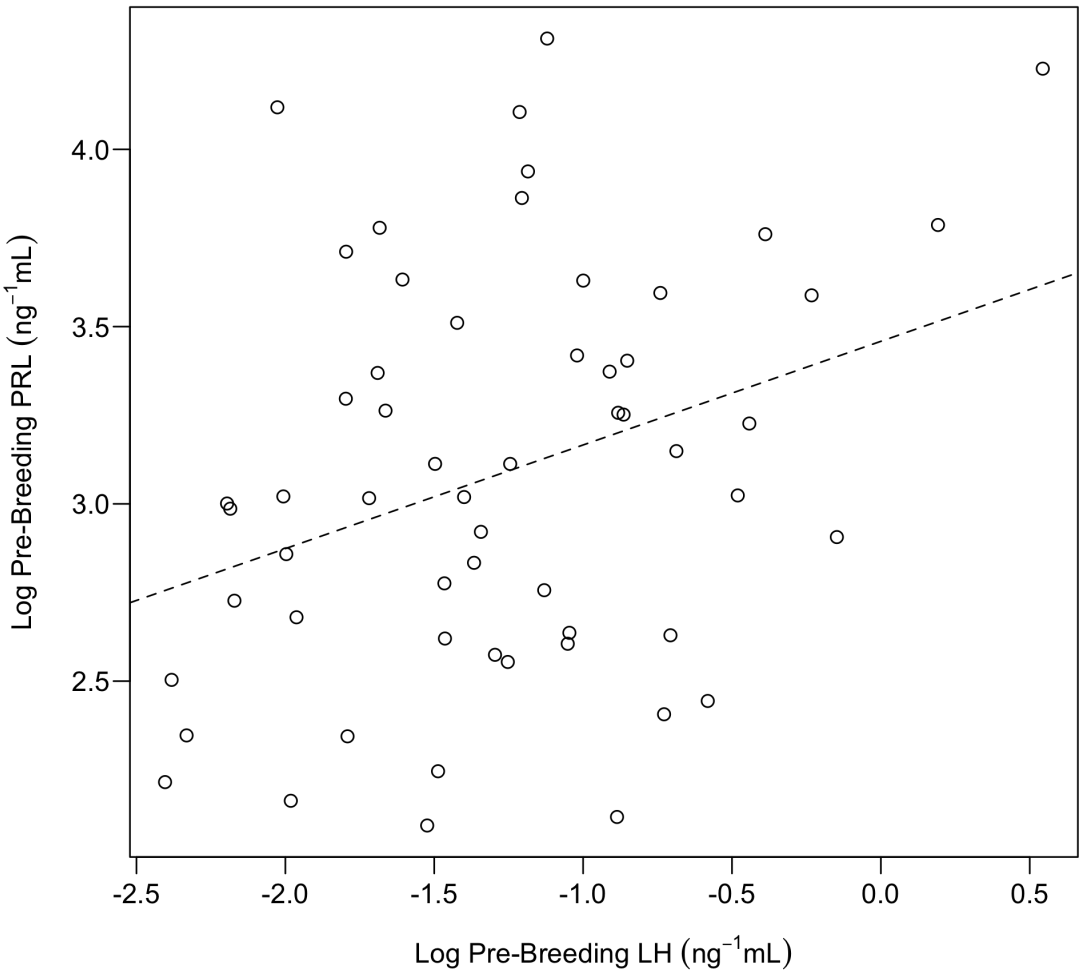

Figure 2.1. ***Relationship (and least squares line) between pre-breeding log prolactin (PRL) and pre-breeding log luteinizing hormone. Correlation between these two traits was significant ($F_{2,54}$ = 7.10,* P = *0.010, ANCOVA model including hematocrit).***

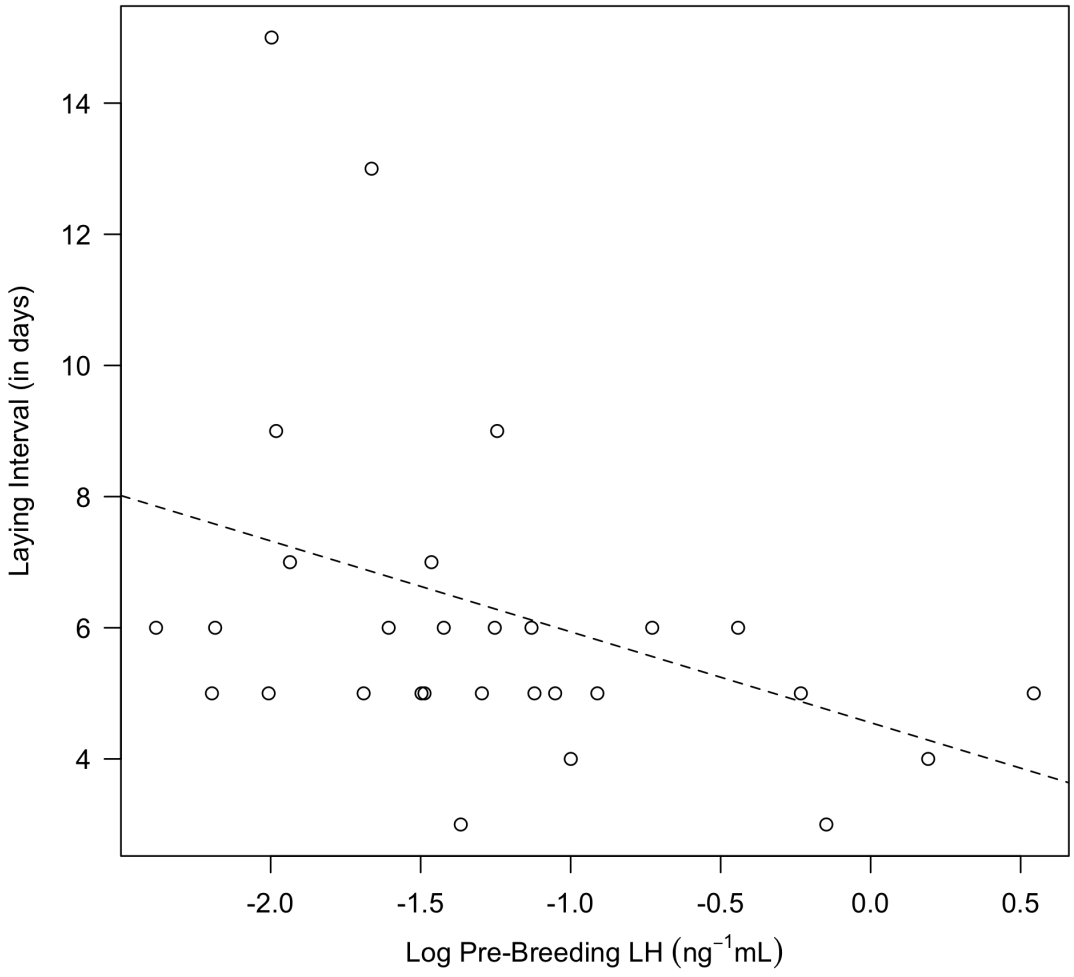

Figure 2.2. ***Relationship between pre-breeding luteinizing hormone (LH) and the interval between pairing and the first egg in subsequent pairing. Females were paired roughly two weeks following pre-breeding blood sampling, and the relationship between log LH and log laying interval was significant ($F_{2,24}$ = 6.67, P = 0.003), controlling for the time between blood sampling and pairing.***

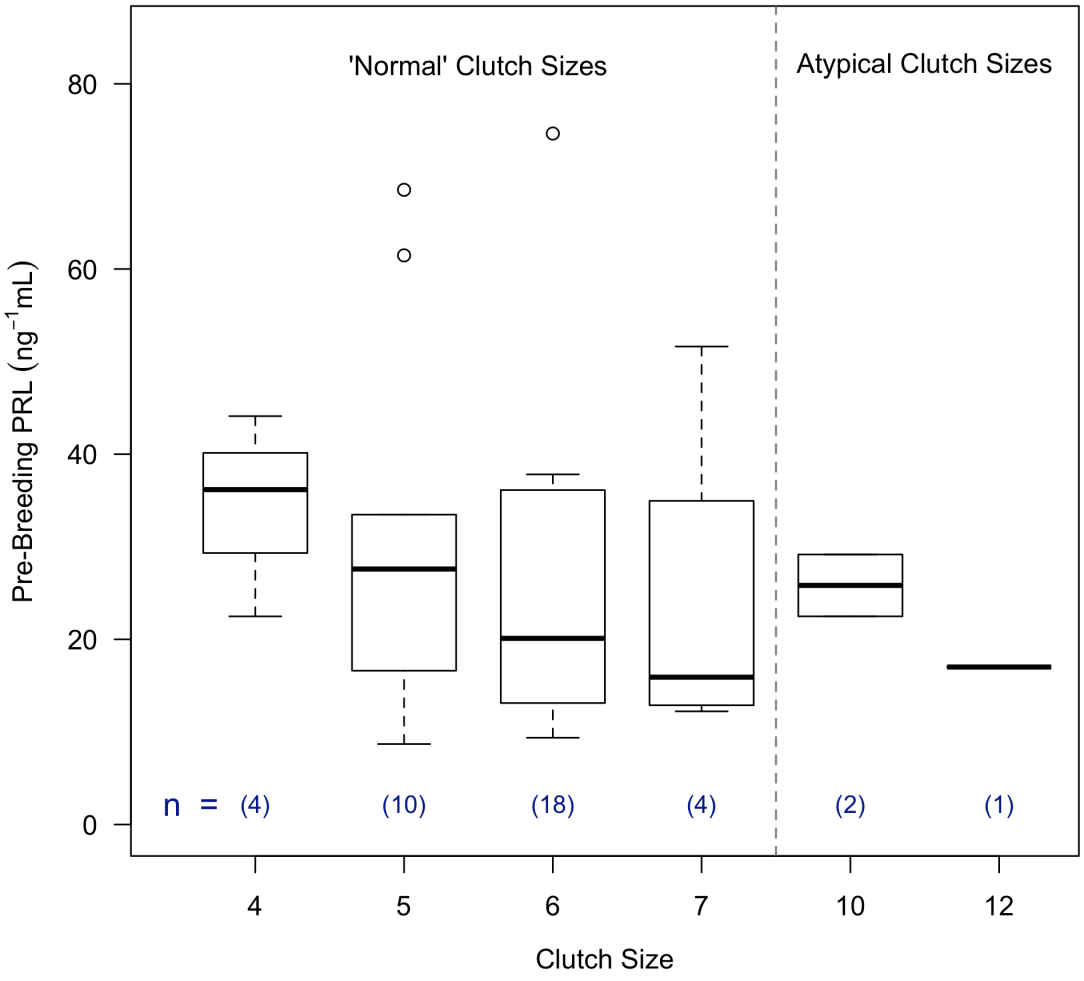

Figure 2.3. ***Pre-breeding prolactin (PRL) in female zebra finches by the number of eggs laid when breeding (approximately two weeks later). Clutch sizes larger than those typically observed in the wild are noted as "atypical", and the number of females laying a given clutch size are indicated in blue. There were no significant differences in pre-breeding log PRL over all observed or normal clutch sizes only.***

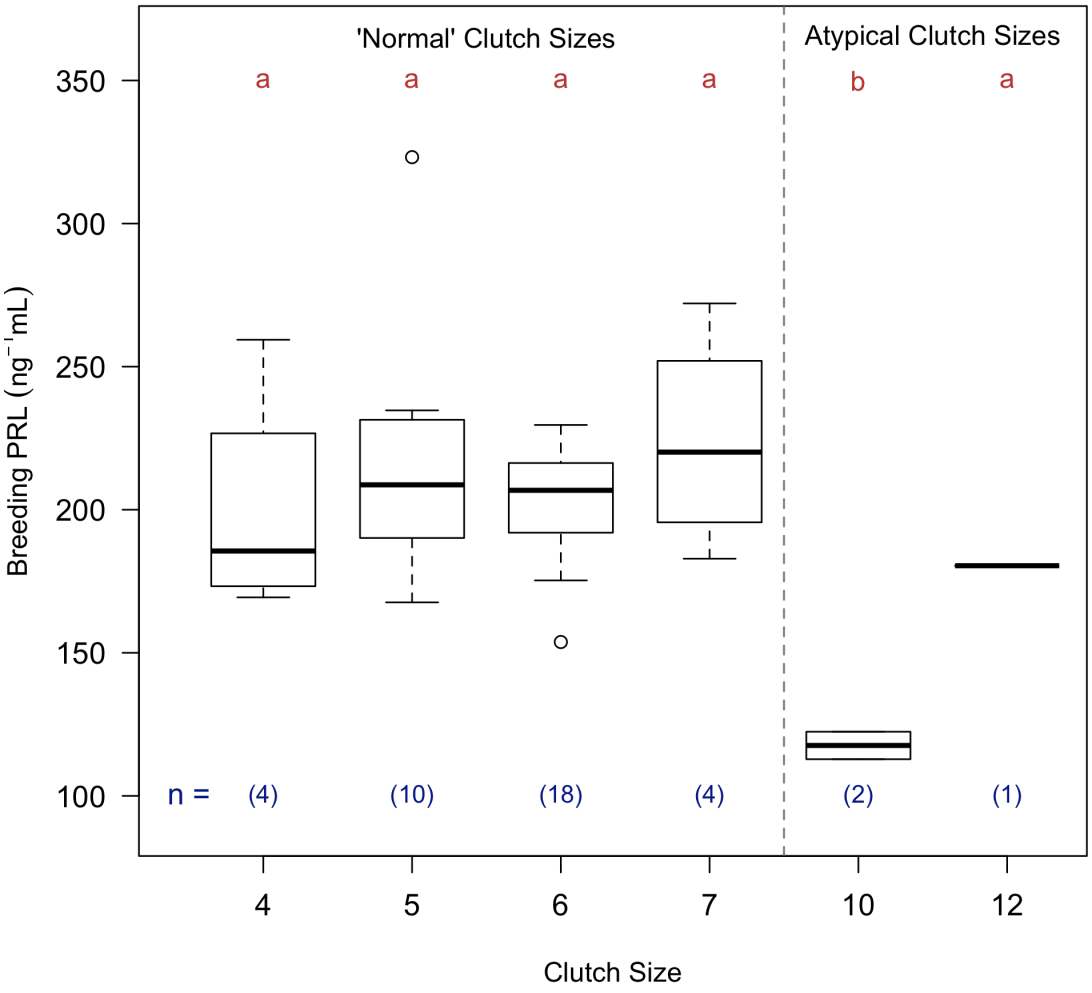

Figure 2.4. ***Breeding prolactin (PRL) in the plasma breeding zebra finch females on the day the third egg was laid. Clutch sizes larger than those typically observed in the wild are noted as "atypical", and clutches sharing the same (red) letters were not significantly different in generalized linear regression (quasipoisson family, logit link). The number of females laying a given clutch size are indicated in blue.***

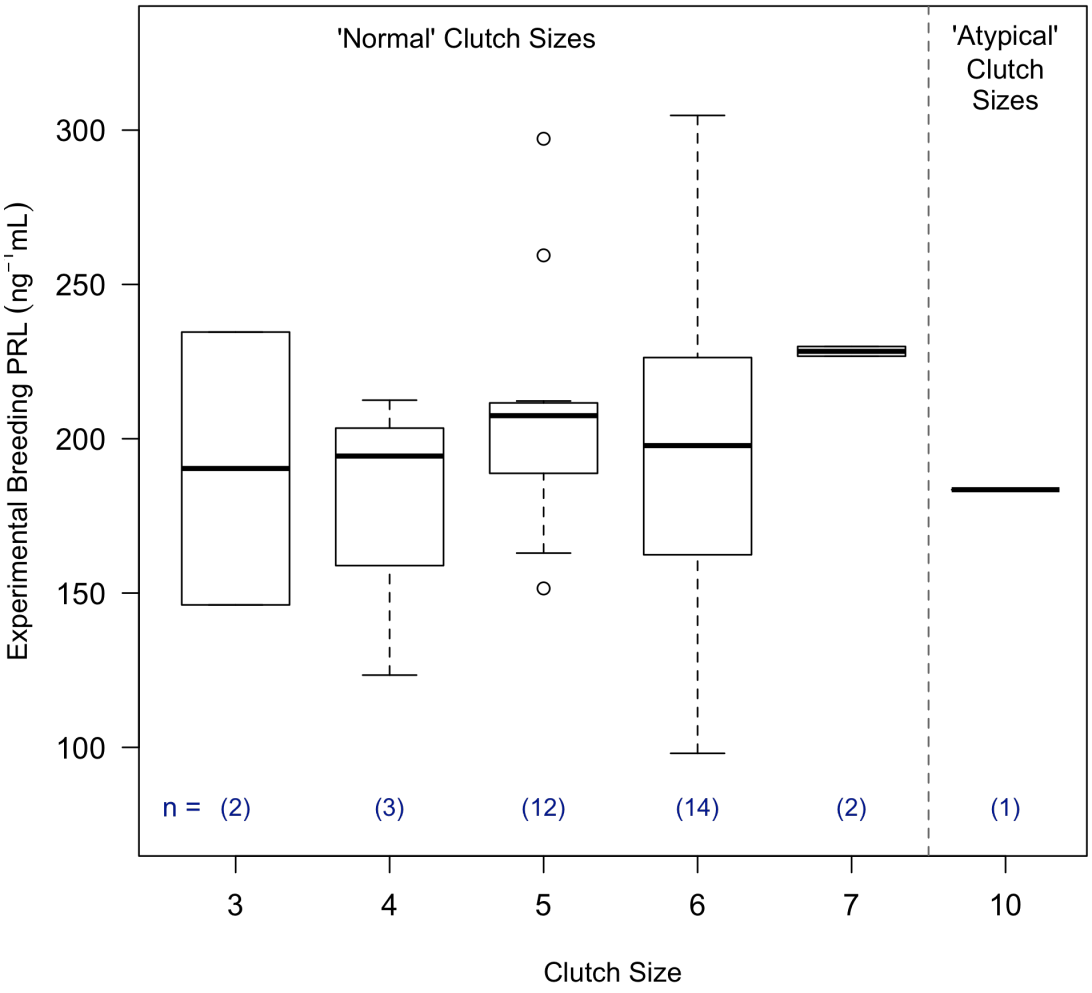

Figure 2.5. ***Breeding prolactin (PRL) in the plasma breeding zebra finch females on the day the third egg was laid during experimental breeding. Females received one of two doses of bromocriptine, or vehicle only. Treatment had no effect on log PRL, nor were there any significant differences in log PRL associated with different clutch sizes. Clutch sizes larger than those typically observed in the wild are noted as "atypical" and the number of females laying a given clutch size are indicated in blue.***

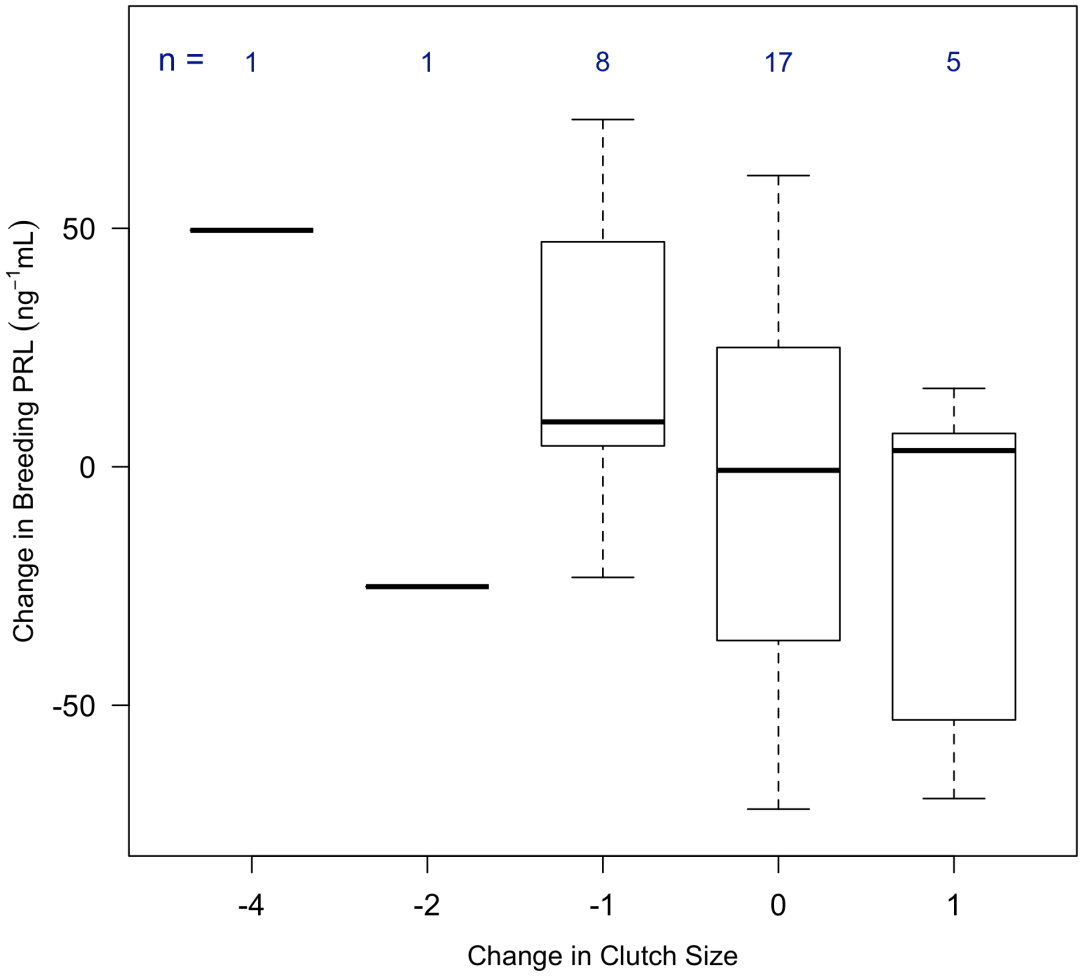

Figure 2.6. ***Change in breeding prolactin (PRL) and clutch size between control and experimental breeding attempts. The relationship between these two traits was significant including (P = 0.031) or excluding (P = 0.041) the two clutches for which there were only one observation (decreases in four and two eggs).***

Chapter 3.

Uncoupling Clutch Size and Plasma Prolactin with Experimental Egg Removal

Calen P. Ryan[1], Alistair S. Dawson[2], Peter J. Sharp[3], and Tony D. Williams[1]

[1]Department of Biological Sciences, Simon Fraser University, 8888 University Drive, Burnaby, British Columbia, Canada, V5A 1S6; [2]Centre for Ecology and Hydrology, Bush Estate, Penicuik, Midlothian, Scotland, U.K., EH26 0QB; [3]The Roslin Institute, University of Edinburgh, Easter Bush, Midlothian, Scotland, U.K., E25 9RG

3.1. Introduction

Clutch size is among the most important contributors to avian lifetime reproductive success, and sets the upper limit on the number of young that can be successfully fledged in any given reproductive event (Rockwell et al. 1987; McCleery et al. 2004; Charmantier et al. 2006). Geographical, temporal, and phylogenetic patterns, as well as sources of individual variability in avian clutch size, have been of great interest to both evolutionary biologists and ecologists. Yet study has focused largely on the selective forces that constrain and shape optimal clutch size (e.g. Lack 1947; Williams 1966; Charnov and Krebs 1974; Pettifor et al. 1988; Rowe et al. 1994; Nager et al. 2000; Martin et al. 2006; Ricklefs 2010), and the social and ecological cues involved in individually fine-tuning that investment under varying conditions (e.g. Lack 1947; Bolton et al. 1993; Williams and Miller 2003; Travers et al. 2010; Zanette et al. 2011; Decker et al. 2012). While progress has been made in these other fields, the physiological and hormonal mechanisms underpinning clutch size determination remain poorly understood (Klomp 1970; Sockman et al. 2006; Williams 2012).

The only physiological or mechanistic hypothesis for avian clutch size determination involves an anti-gonadal effect of prolactin (PRL), a peptide hormone that stimulates, and is stimulated by, incubation behaviour (El Halawani et al. 1984; March et al. 1994; Delehanty et al. 1997) and chick rearing (O'Dwyer et al. 2006; Angelier and Chastel 2009; Miller et al. 2009). This hypothesis is built around a number of well-supported observations, namely: a) a close temporal association between the development of incubation behaviour and the cessation of laying (Lea et al. 1981; Haftorn 1981); b) a positive feedback loop between the development of incubation behaviour, tactile stimulation from the eggs, and plasma PRL levels (Lea et al. 1981; El Halawani et al. 1984), and; c) a seasonal increase in the rate of incubation onset and plasma PRL during laying, and a seasonal decline in clutch size (Meijer et al. 1990; Flint et al. 2006). Collectively, these observations provide support for a mechanistic model whereby PRL regulates clutch size, and is itself mediated through seasonal changes in PRL and the onset of incubation behaviour (Murton and Westwood 1977; Haftorn 1981; Meijer et al. 1990; Haywood 1993a; Sockman et al. 2006). Unfortunately, much of the data supporting a mechanistic role for PRL in clutch size determination has been derived

from correlational studies, which rely heavily on broad temporal associations between incubation, clutch size, and changes in circulating PRL rather than direct experimental support. The other component of the PRL-based mechanistic model for clutch size determination involves an anti-gonadal effect of PRL, potentially via inhibition of luteinizing hormone (LH), yet evidence for this effect *in vivo* is lacking. Potential indirect anti-gonadal effects of PRL via LH have been demonstrated in *in vitro* assays in poultry (El Halawani et al. 1984; Rozenboim et al. 1993; You et al. 1995), and are supported by evidence for anti-gonadal effects of PRL *in vivo* in some species (Bailey 1950; Meier 1969; Reddy et al. 2007), but not others (Meier and Dusseau 1968; Lea et al. 1991; Buntin et al. 1999; Small et al. 2007; Ryan et al., In prep). Therefore, whether or not experimentally manipulated PRL levels, let alone natural individual variation in PRL, have the capacity to inhibit or disrupt follicle growth and directly affect clutch size has not yet been demonstrated.

Experimental work directly examining the role of PRL in clutch size determination in non-domesticated species laying discrete clutches (i.e. retaining cyclic reproduction characteristic of wild birds) is limited to two studies (Sockman et al. 2000; Ryan et al. In prep). In one study, Sockman and colleagues (2000) found weak support for a negative association between clutch size and PRL at the time when follicular inhibition putatively occurs. However, PRL manipulations using ovine-PRL osmotic minipumps were unsuccessful and were not associated with changes in clutch size (Sockman et al. 2000). A more recent study in captive-breeding zebra finches (*Taeniopygia guttata*) also found no support for a relationship between plasma PRL (measured at day 3 of egg-laying) and clutch size, nor was there evidence for an inhibitory, anti-gonadal, effect of PRL on LH (Ryan et al. In prep). However, in the latter study, pharmacological suppression of PRL using bromocriptine was unsuccessful, so experimental evidence for or against the PRL-based mechanism for clutch size determination is still lacking.

In this study we take a complimentary approach to that reported in Ryan et al. (In prep.). To experimentally test predictions of the PRL-based mechanism for clutch size determination in zebra finches (*Taeniopygia guttata*), we used egg-removal to increase clutch size and assessed whether circulating PRL and LH levels (i.e. the hormones putatively mediating clutch size) responded to this experimental manipulation. Based on the current mechanistic model for clutch size determination (reviewed in Sockman et al.

2006), we had a series of *a priori* predictions. We predicted: a) that egg-removal females would have lower plasma PRL and higher LH early in laying (the day the 3rd egg is laid), given that egg-removal increases clutch size and that the signal inhibiting follicle growth in *T. guttata* is postulated to occur invariably at this time (Haywood 1993c), and; b) a negative relationship between plasma PRL on egg day 3 and final clutch size, regardless of individual variation in response to egg removal (Williams & Miller 2003), again based on the temporally invariable inhibition of clutch size. If, contrary to Haywood (1993c) the inhibitory signal for clutch size determination in zebra finches varies temporally as in other species (e.g. *Parus caeruleus*; Haywood 1993b), we considered the possibility that egg removal could be delaying the timing of follicular inhibition. Under this scenario, we alternatively predicted: c) a negative relationship between plasma PRL at later time points (egg days 10 or 17) and clutch size, for females who responded to egg removal by laying very large clutches.

These predictions are based on absolute PRL levels at a single time point (either egg day 3, or for females who continued laying, egg days 10 and 17). However, for females with multiple PRL measurements, we predicted that females with the most rapid increase in plasma PRL would reach a 'threshold' level for follicular inhibition sooner (Williams 2012) and so would lay fewer additional eggs compared to other females within the same range of clutch size. In other words, we predicted that a female with a rapid increase in PRL between the 3rd and 10th egg would lay fewer additional eggs compared to a female with a relatively gradual increase in PRL during this same time, even though clutches greater than 10 eggs exceed those normally observed in the wild (2-7 eggs; Zann 1996). Finally, since we had breeding data for all females for a pre-treatment clutch before the egg removal experiment, we were able to test similar predictions about *changes* in PRL and clutch size within individual females across the two breeding attempts. Thus, our repeated-measures experimental design allowed us to analyze individual variability and plasticity in hormone-trait (i.e. PRL-clutch size) relationships across time or experimental conditions, i.e. to consider physiological data from the 'reaction norm' perspective more commonly employed in ecological studies (Nussey et al. 2007; Williams 2008).

3.2. Methods and Materials

3.2.1. Animal care and breeding protocol

Zebra finches were maintained in controlled environmental conditions (temperature 19–23C; humidity 35–55%; constant light schedule, 14 L:10 D, lights on at 07.00). All birds were provided with a mixed seed diet (*Panicum* and white millet, 1:1, 11.7% protein, 0.6% lipid and 84.3% carbohydrate by dry mass), water, grit and cuttlefish bone (calcium) *ad libitum*, and received a multi-vitamin supplement in the drinking water once per week. Breeding pairs were also provided with 6 g/pair per day of an egg food supplement (20.3% protein, 6.6% lipid) between pairing and clutch completion. Prior to the experiment, all birds were housed in same-sex cages (61cm x 46cm x 41cm) but were not visually or acoustically isolated from the opposite sex. At the beginning of the experiment, all individuals were 4-10 months of age, had been successfully bred at least once, and were always paired with the same individual of the opposite sex to minimize variation in investment based on perceived mate quality. As far as possible, the same individuals were used for both breeding events, so that intra-individual comparisons (i.e. reaction norms) could be made between the 'Pre-treatment' and 'Treatment' breeding events. Breeding pairs were housed individually in cages (61cm x 46 cm x 41 cm), each with an external nest-box (11.5cm x 11.5cm x 11.5cm). Females were weighed (± 0.1 g, initial mass) at the time of pairing, just prior to blood sampling, and at clutch completion. Nest-boxes were checked daily between 09.30 and 11.30 and all new eggs were weighed (to 0.001 g) and numbered to obtain data on egg size, clutch size and laying interval (the time between pairing and laying of the first egg).

3.2.2. Experimental Protocol

During the 'Pre-treatment' breeding, eggs were immediately returned to the nest in which they were laid after measurement (i.e. there was no egg removal). For the subsequent 'Treatment' breeding, females were assigned either to 'Egg-removal' or 'Untreated control' groups (Fig. 3.1). For egg removal females, eggs 1 through 14 were removed from the nest as they were laid and were not returned, to induce continued laying. To look at the effect of egg contact after continued laying, eggs 15 and onwards for Egg-removal females were no longer removed, but were allowed to accumulate

normally in the nest until clutch completion (Fig. 3.1). For untreated control females in the Treatment breeding, eggs were immediately returned to the nest in which they were laid after weighing (as for Pre-treatment clutches). For both treatment groups and breeding attempts, a clutch was considered complete when no additional eggs were produced over two consecutive days. At clutch completion, individuals were returned to same-sex holding cages for a recovery period of at least three weeks before repairing. Experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (no. 901B 94), in accordance with guidelines from the Canadian Committee on Animal Care (CCAC).

3.2.3. Hormone assays

For the Pre-treatment breeding, all females were blood sampled (≤200 µL, from the brachial vein) on the day the 3rd egg was laid. Egg day 3 was selected based the experimental work which links the physiological mechanism for clutch size determination in zebra finches invariably with the timing of the 3rd laid egg (Haywood 1993b; see Discussion). Blood samples were also taken on the day the 3rd egg was laid for both Egg-removal females and controls in the Treatment breeding. For the Treatment breeding, extending the laying period using egg removal allowed us to take additional blood samples from Egg-removal on the days the 10th and 17th eggs were laid or at clutch completion if this occurred within one day of the 10th or 17th egg. For Egg-removal females, all three blood samples were separated by roughly 7 day intervals, and allowed us to look at PRL and LH levels in the absence of eggs in the nest within normal (2-7 eggs) and supra-normal clutch size (10 eggs). By leaving the 15th, 16th, and 17th eggs, and blood sampling on egg day 17, we were also able to look at PRL and LH well-beyond the normal clutch size, but still with only 3 eggs physically present in the nest. Although our sample sizes were considerably reduced by this time, this last blood sample allowed us to make hormone comparisons with Pre-treatment breeding and control females (when three eggs had also accumulated in the nest). Blood sampling was carried out between 1130 and 1330, predicted to be the exact window of time where follicular inhibition occurs (Haywood 1993c), and to minimize circadian fluctuations in hormone levels. Blood was thereafter centrifuged at 13,000 rpm for 5 minutes, and plasma was stored at –20°C until required for hormone assays.

Plasma immunoreactive prolactin (PRL) was determined using a radioimmunoassay for recombinant-derived Starling (*Sturnus vulgaris)* PRL described by Bentley et al. (1997). Samples were measured in duplicate in a single assay, diluted 1 in 3. The sensitivity of the assay, determined to be the estimated concentration two standard deviations above the mean counts per minute of the lowest standard, was 7.779 $ng \cdot mL^{-1}$, after correcting for dilution. Samples with concentrations below this value were not included in analyses. The intra-assay coefficient of variation of this assay was 6.5%, and serial dilution of individual samples ran parallel along the standard curve within the range assayed. Luteinizing hormone (LH) was measured using a micro-modified version of a previously described radioimmunoassay (Sharp et al. 1987). Samples were run in a single assay, in duplicate when sample volume permitted (>90% of all samples), diluted 1 in 2.3 in radioimmunoassay (RIA) buffer. Assay sensitivity was determined as described above, with a lower limit of 0.087$ng \cdot mL^{-1}$, after correcting for dilution. Samples with concentrations below this value were not included in analyses. The intra-assay coefficient of variation for this assay was 6.4% for a high value pool and 8.1% for a low value pool, and a curve generated by serial dilution of Zebra finch plasma ran parallel to the standard curve within the range assayed.

3.2.4. Statistical analyses

Data were first examined for normality, outliers, collinearity and interactions between explanatory variables (Zuur et al. 2010). Only LH showed deviations from normality, which was corrected by log transformation. Since there were no statistical differences in the results found using mass alone or the residuals of a regression of mass by tarsus, mass alone was used as a covariate in all relevant analyses. Simple comparisons (excluding clutch size; see below) were conducted using ANOVA or ordinary least squares regression. For repeated measures analysis we used linear mixed effects models with individual female as a random factor using the statistical package 'nlme' in R 2.12.2 (R Core Development Team 2011; Pinheiro et al. 2011). Pre-treatment breeding sample size was 44 pairs, while Treatment breeding sample size was 39 pairs (27 egg removal pairs; 12 control pairs). In both the Pre-treatment and Treatment breeding attempts, a subset of females were not available or failed to breed, laid fewer than 3 eggs (i.e. no hormone values for egg day three), did not provide

sufficient plasma for both hormone assays, or had hormone values below the detection limit (described above). Also, since individual response to egg removal treatment differed, only a subset of females who laid more than 3 eggs were still laying for the egg 10 blood sample, and only a subset who laid more than 10 eggs were still laying for the 17 egg blood sample. As a result, model degrees of freedom vary, particularly for between treatment comparisons.

Since clutch size is a discrete count variable, all analyses of this trait were conducted using generalized linear or generalized linear mixed effects models, with quasipoisson family to account for underdispersion (R package "glmmPQL"; Fox and Weisberg 2011). Analyses of egg mass were conducted on mean egg mass per clutch. Analyses were always followed with standard model validation procedures to test the assumptions of the test employed. Data points with high leverage and Cook's distance (greater than 4/n) were considered influential, and outputs are presented for models including and excluding these points for transparency. Repeatability was calculated using methods previously described (Lessells and Boag 1987).

3.3. Results

Mass at pairing increased from 14.62 ± 0.20 g to 15.32 ± 0.16 g ($P < 0.001$) and laying interval decreased from 5.9 ± 1.1 days to 4.1 ± 1.1 days ($P < 0.001$) between Pre-treatment and Treatment breeding attempts. However, there were no differences in these traits for females assigned to Egg-removal or Control treatments for either the Pre-treatment or Treatment breeding attempts ($P > 0.15$). There was also no difference between Egg-removal and Control females in Pre-treatment clutch size ($P > 0.50$).

3.3.1. Effect of egg removal on clutch size

In the Treatment breeding, Egg-removal females laid significantly larger clutches than Control females ($P < 0.001$). Between the Pre-treatment breeding and Treatment breeding attempts, Egg-removal females increased their clutch size significantly from 5.3 ± 0.7 eggs to 13.2 ±1.9 eggs ($F_{1,24} = 72.78$, $P < 0.001$; Fig. 3.2), whereas Control females did not (5.9 ±0.6 eggs to 5.6 ± 0.7 eggs, respectively; $F_{1,11} = 0.43$, $P > 0.5$; Fig.

3.2). There was marked individual variation in changes in clutch size in response to egg removal: females laid from 3 fewer to 15 additional eggs during their Treatment clutch compared with their Pre-treatment clutch (Fig. 3.2). Change in clutch size in Control females varied from 3 fewer to 1 additional egg (Fig. 3.2). Variation in Treatment clutch size in Egg-removal females was dependent on Pre-treatment clutch size and egg mass. Females who laid the largest clutches ($F_{1,23}$ = 4.80, P = 0.039) and who had largest mean egg mass ($F_{1,23}$ = 8.96, P < 0.001) during the Pre-treatment breeding went on to lay the largest clutches in response to egg removal. To investigate potential effects of differences in 'quality' or efficiency in reproductive investment, we examined changes in mass between pairing and laying. Mass at pairing was significantly correlated with changes in mass between pairing and clutch completion for both Pre-treatment and Treatment breeding attempts, i.e. heavier females lost the most weight throughout laying ($F_{1,39}$ = 21.88 and $F_{1,34}$ = 18.62 , both P < 0.001). However, for both Pre-treatment and Treatment breeding attempts, mass loss was independent of treatment ($F_{2,33}$ = 0.56 and $F_{2,33}$ = 4.03; P > 0.05 for both) and final clutch size ($F_{2,38}$ = 0.01 and $F_{2,33}$ = 0.49; P > 0.4) after controlling for mass at pairing.

3.3.2. Effect of egg removal on PRL and LH early in laying

There were no significant differences in day 3 plasma PRL between Egg-removal and Control females for either the Pre-treatment ($F_{1,32}$ < 0.01, P = 0.978) or the Treatment breeding attempts ($F_{1,30}$ = 1.29, P = 0.265). Standard model validation procedures of the analysis examining treatment (egg-removal versus control), breeding attempt (Pre-treatment versus Treatment), and the interaction between them revealed one particularly low PRL value for a Control female in the Pre-treatment breeding to be highly influential on the outcome of the model (grey dashed-line; Fig. 3.3). PRL decreased significantly between Pre-treatment and Treatment breeding attempts (from 204.5 ± 5.6 ng·mL^{-1} to 165.7 ± 8.2 ng·mL^{-}1; $F_{1,27}$ = 33.19, P < 0.001; Fig. 3.3), but there was no significant treatment by breeding attempt interaction after removing this high leverage data point ($F_{1,27}$ = 0.70, P = 0.41). There were no significant differences in day 3 plasma LH between Egg-removal and Control females for either the Pre-treatment ($F_{1,21}$ = 1.97, P = 0.175) or the Treatment breeding attempts ($F_{1,27}$ = 1.88, P = 0.181). LH did not significantly change between the Pre-treatment and Treatment breeding

attempts, nor was there a significant treatment by breeding attempt interaction ($F_{1,21}$ = 1.62, P = 0.217).

3.3.3. Relationship between PRL, LH, and final clutch size with extended laying

In the Treatment (egg removal) experiment, final clutch size was independent of day 3 PRL (controlling for treatment type; ANCOVA: $F_{2,29}$ = 0.46; P = 0.50; Fig. 3.4) and LH ($F_{2,26}$ = 0.17; P = 0.708). For Egg-removal females, final clutch size was also independent of plasma PRL on egg days 10 ($F_{1,17}$ = 1.99; P = 0.18) and egg days 17 ($F_{1,7}$ = 3.27; P = 0.114). Final clutch size was also independent of LH on days 10 ($F_{1,14}$ = 1.31; P = 0.27) and 17 ($F_{1,6}$ = 1.01; P = 0.353). During the Treatment breeding, circulating PRL increased in Egg-removal females between egg days 3 (158.5 ±9.0ng·mL^{-1}) and 10 (183.2 ± 8.8 ng·mL^{-1}; $F_{1,16}$ = 5.50; P = 0.032), and egg days 10 and 17 (213.7 ± 11.3 ng·mL^{-1}; $F_{1,8}$ = 16.40; P = 0.004; Fig. 3.5). Plasma PRL concentrations between days 3 and 10 of egg removal were positively correlated ($F_{1,15}$ = 7.77; r^2 = 0.30; P = 0.014; Fig. 3.5), but there was no intra-individual correlation in PRL levels between days 10 and 17 or between days 3 and 17 ($F_{1,6}$ = 1.50 and $F_{1,7}$ = 2.08; P > 0.10 for both; Fig. 3.5). In contrast to plasma PRL, plasma LH on egg day 17 (0.27 ± 0.08 ng·mL^{-1}) was significantly *lower* than on egg days 3 (0.48 ± 0.07 ng·mL^{-1}; $F_{1,7}$ = 15.83; P = 0.005) and 10 (0.52 ± 0.08 ng·mL^{-1}; $F_{1,6}$ = 7.71; P = 0.032) for egg removal females. Plasma LH levels were not correlated between days 3 and 10 ($F_{1,12}$ = 1.05, P = 0.326), or 10 and 17 ($F_{1,6}$ = 4.21, P = 0.086). There were no significant correlations between plasma PRL and plasma LH concentrations for day 3, 10 or 17 for either Egg-removal or Control females (P > 0.3 for all).

3.3.4. Relationship between changes in PRL and LH during extended laying and final clutch size

To investigate relationships between individual rate of change in plasma PRL within the Treatment breeding and its relationship to final clutch size, we calculated individual slopes from the difference in PRL between egg days 3 and 10 and egg days 10 and 17 (see Fig. 3.5). Final clutch size was independent of the change in plasma PRL between the two time points nearest to clutch completion (days 3 to 10 or 10 to 17; $F_{1,15}$

= 0.45; $P > 0.5$). Final treatment clutch size was also independent of the change in plasma LH between these same two time points (days 3 to 10 or 10 to 17; $F_{1,14} = 0.346$; $P > 0.5$). Furthermore, there were no significant correlations between the magnitude and direction of change in PRL and the magnitude and direction of change in LH between days 3 and 10 or 10 and 17 (using the value for the slope closest to clutch completion; $F_{1,15} = 0.54$, $P = 0.472$).

3.3.5. Relationship between changes in PRL and LH and changes in clutch size between breeding attempts

Between the Pre-treatment and Treatment breeding, individual variation in the magnitude and direction of changes in clutch size were not associated with the magnitude and direction in changes in PRL ($F_{1,27} = 0.12$; $P = 0.730$) or LH ($F_{1,21} = 0.92$; $P = 0.348$). In other words, changes in these hormones did not correspond to changes in clutch size between breeding attempts. There was also no relationship between changes in day 3 PRL between the Pre-treatment and Treatment breeding and changes in day 3 LH during this same time period ($F_{1,21} = 0.01$; $P = 0.914$). While day 3 plasma PRL was significantly lower during the Treatment breeding than the Pre-treatment breeding, there were no significant differences in PRL on days 10 and 17 of the Treatment breeding compared to day 3 of the Pre-treatment breeding ($F_{1,15} = 3.23$; $P = 0.093$ and $F_{1,8} = 3.40$; $P = 0.102$). Similarly, LH on egg day 17 of the Treatment clutch was significantly lower than egg day 3 for the Pre-treatment clutch (0.51 ± 0.05 ng·mL^{-1}; $F_{1,6} = 7.34$; $P = 0.035$).

3.3.6. PRL and LH and time to clutch completion

Finally, we analyzed plasma PRL concentration for the blood sample closest to the time of clutch completion in relation to subsequent duration of egg laying. There was a significant negative relationship between plasma PRL prior to clutch completion and the time in days to clutch completion for the Treatment breeding ($F_{1,31} = 4.47$, adjusted $R^2 = 0.10$, $P = 0.043$). Looking at treatment groups separately, this relationship remained significant for egg removal females ($F_{1,20} = 6.04$, adjusted $R^2 = 0.194$, $P = 0.023$; Fig. 3.6) but not controls ($F_{1,9} = 0.11$, $P = 0.746$). The relationship between PRL and the time in days to clutch completion was also significant for the Pre-treatment breeding, but with no significant treatment effect ($F_{2,31} = 5.91$, $P = 0.021$ for days to completion, and $F_{2,31} =$

0.06, P = 0.81 for treatment). Several females naturally laying large (> 8 egg) clutches in the range of those observed in the Treatment breeding had a strong influence on the outcome of the Pre-treatment breeding analysis. Plasma LH levels closest to the time to clutch completion were not associated with the time in days to clutch completion for the Treatment breeding ($F_{1,25}$ = 0.433, P = 0.516), including when examining each treatment group separately (P > 0.4 for both). There was also no relationship between LH and time to clutch completion for the Pre-treatment breeding, and no effect of treatment ($F_{2,27}$ = 0.077, P = 0.784 for days to completion, and $F_{2,27}$ = 0.014, P = 0.906 for treatment).

3.4. Discussion

The objective of this study was to test experimentally the PRL-based mechanistic model for clutch size determination in captive-breeding zebra finches, using an approach complimentary to that reported in Ryan et al. (In prep.). Egg removal resulted in significantly larger clutch sizes, but with considerable individual variability in response to treatment. Despite this, the large variation in manipulated clutch size was not associated with significant differences in circulating levels of either PRL or LH, between the Egg-removal and Control females, at the time when follicular inhibition in zebra finches is postulated to occur (day 3 of egg-laying; Haywood 1993c). Individual differences in plasma PRL on egg day 3 also did not predict final clutch size, nor did individual PRL on days 10 or 17. Plasma PRL concentrations increased, and LH decreased, through extended laying, but the individual rate of change in these hormones over time was not predictive of final clutch size. Similarly, although circulating PRL decreased in individual females between the Pre-treatment and Treatment breeding, this change was not associated with individual changes in clutch size. PRL levels at the time closest to clutch completion were, however, negatively correlated with the number of laying days remaining for all females in the Pre-treatment breeding, and Egg-removal females but not controls in the Treatment breeding.

3.4.1. Relationship between Treatment breeding PRL, LH and clutch size

Clutch sizes for Egg-removal females were significantly larger than those of controls, consistent with previous studies in zebra finches (Haywood 1993c; Williams and Miller 2003), validating our experimental approach. In contrast, plasma PRL levels on day 3 of the Treatment breeding were not significantly different between Egg-removal females and controls, in contrast to predictions of the mechanistic model for clutch size determination. Similarly, there was no effect of egg removal on plasma LH at this time. Breeding PRL levels are highly variable (Maney et al. 1999; Schaper et al. 2012; Ryan et al. In prep; this study), but follicular inhibition controlling clutch size in zebra finches is postulated to occur invariably on the day the third egg is laid (Haywood 1993c). Nonetheless, in our study PRL on the day the third egg was laid did not explain clutch size significantly for egg removal females or controls during the Treatment breeding. Moreover, PRL on the days the 10^{th} and 17^{th} eggs were laid also were not associated with final clutch size, though the power of our ability to detect effects is reduced for these later time points. Similar results were found for LH. In contrast, Millam et al. (1996) reported a significant relationship between PRL 17 days into incubation and clutch size in canaries, but this time point included values for females who had already completed laying (Millam et al. 1996). Since PRL increases rapidly with incubation and clutch completion (Sharp et al. 1998), and the only period in which PRL could exert regulatory control over clutch size is between the recruitment and ovulation of the last follicle (Sockman et al. 2006), females that had already finished laying prior to the 17^{th} day of incubation in that study (i.e. smaller clutch sizes) were unnecessarily included in their analysis (Millam et al. 1996). Accordingly, PRL during the 2^{nd}, 7^{th}, and 12^{th} days of incubation in that study (when all females were still laying) showed no relationship with clutch size (Millam et al. 1996). In the current study, all samples were taken from females who were still laying, and we similarly found no relationship between PRL and clutch size.

3.4.2. Relationship between changes in PRL and LH during extended laying and final clutch size

PRL at several time points (days 3, 10 and 17) through laying were not predictive of final clutch size. However, individuals can vary in rates of hormonal change in response to breeding stimuli. For example, different populations of White-Crowned sparrows (*Zonotrichia leucophrys*) exposed to long-day photoperiodic cues exhibit marked individual variation in the rate of development of the brood patch and ovaries (Lewis 1975) and the rate of increases in plasma LH (Wingfield et al. 1980). Similar individual variability in our captive-breeding zebra finches is supported by the variability in the interval between pairing and laying the first egg and its hormonal correlates (Ryan et al. In prep). Indeed, such 'fluctuating signals' could be informative in and of themselves (Williams 2008), particularly if they follow a predictable pattern. While all plasma samples were taken at the same time of day to minimize circadian fluctuations, we considered individual differences in the rate of change in PRL and LH between egg days 3 and 10 or 10 and 17 to study these potentially informative fluctuating signals. We predicted that the steepest increase in PRL would be associated with a rapid attainment of the critical inhibitory threshold value for PRL (Sockman et al. 2006), and the fewest additional eggs laid after that point. Nonetheless, we observed no significant association between rate of PRL increase and total clutch size, nor between rate of increase and the number of additional eggs laid. Consistent with the other findings of this study, these results fail to support for a role for PRL in clutch size determination.

3.4.3. Changes in PRL, LH and clutch size between Pre-treatment and Treatment breeding attempts

Using data from a previous breeding attempt, we were able to examine covariation in changes in PRL, LH and clutch size among breeding attempts. Between the Pre-treatment and Treatment breeding, the majority (84%) of females responded to egg removal by increasing clutch size, but we also observed marked individual variability in this response. This variability was not predicted by individual mass or condition at the time of breeding, and there were no differences in mass loss during laying by treatment group or clutch size. Additionally, our females all had access to *ad libitum* feed and a high-protein egg laying supplement, so our results probably do not reflect differences in

resource availability (Williams and Miller 2003; Gorman and Nager 2003). Rather, this variability in response suggests differences in individual 'quality' or allocation strategies (Charnov and Krebs 1974; Hamel et al. 2009; Lescroël et al. 2009). Consistent with individual differences in quality, we found that individual variation in total clutch size during the Treatment breeding was predicted by individual variation in Pre-treatment clutch size and egg mass. Variability in clutch size for Egg-removal females is therefore effectively an extension of the natural variability already present in un-manipulated laying zebra finches (Williams 1996), which is integral to our experimental approach to testing the PRL-based mechanism for clutch size determination.

Between the Pre-treatment and Treatment breeding, plasma PRL levels on day 3 of egg-laying decreased between the two breeding attempts, but in contrast to clutch size, the decrease did not differ significantly between Egg-removal and Control treatment groups. There was no change in LH between the Pre-treatment and Treatment breeding attempts for either egg removal or control groups. Decreases in PRL and increases in clutch size *for the egg removal group* agree broadly with predictions of the PRL-based mechanistic model for clutch size determination, but decreases in PRL in the absence of increases in clutch size *for the control group* do not. For egg removal females, we predicted decreases in PRL between the two breeding attempts similar to the ones we observed, due to the lack of tactile stimulation from the eggs during laying. Reductions in plasma PRL have been associated with anaesthetization (Hall and Goldsmith 1983) and denervation (Book et al. 1991) of the brood patch, and increases in PRL have been associated with the addition of artificial eggs prior to laying (Massaro et al. 2007). However, we also predicted significant differences in this change in PRL between Egg-removal and Control females, since the latter did receive tactile stimuli from eggs in the nest. Several factors may explain the lack of such differences. First, plasma PRL increases gradually with the onset of laying, followed by rapid increases late in laying as females approach clutch completion (Hall and Goldsmith 1983; Buntin et al. 1996). Our measurements relatively early relative to clutch completion may have captured the endogenous increase accompanying laying, but not the rapid increase stimulated by tactile stimulation from the eggs. In support of this idea, a previous study failed to detect differences in PRL between laying female cockatiels (*Nymphicus hollandicus*) who had some eggs removed compared to controls (Millam et al. 1996).

However, significant differences in PRL were observed between treatment groups in that study after the majority of females had finished laying (Millam et al. 1996). Furthermore, to stimulate larger than normal clutch sizes in Egg-removal females, we removed eggs but not the nest itself (cf. Buntin et al. 1996). Although speculative, time spent engaged in nesting behaviour even in the absence of eggs may have attenuated any effect of egg removal on plasma PRL in our study, since even the empty nest can contribute to broody behaviour and elevated PRL (Zadworny et al. 1988). Such an attenuated response to egg removal would manifest as reduced differences in PRL between Egg-removal females and controls as observed in this study. There are also examples where tactile stimulation from the nest or eggs is not necessary to maintain elevated PRL levels (i.e. through extended foraging in seabirds, Hector and Goldsmith 1985; i.e. between broods in multiple-brood species, Hiatt et al. 1987). In addition, age-related declines in PRL accompanying the declining ability to successfully raise chicks have been suggested in other species (Angelier et al. 2006), and could explain the decrease we observed in PRL for both control and treatment groups; although our females were only 3 months older, on average, during the Treatment breeding attempt. Regardless of the underlying causes, changes in clutch size were not associated with changes in PRL at the level of the treatment, contrary to the broad predictions of the PRL-based mechanistic model.

3.4.4. Relationship between changes in PRL and LH and changes in clutch size between breeding attempts

The marked individual variation in changes in clutch size and PRL between the two breeding attempts, particularly for females with eggs removed, could make looking at *individual* changes (i.e. plasticity) in PRL, LH and clutch size a useful compliment to group level comparisons. Considering physiological data as 'reaction norms' may better reflect hormone-trait relationships and individually-variable strategies (Vézina et al. 2006; Williams 2008; Ryan et al. In prep). We therefore examined the relationship between the magnitude and direction of *individual* changes in clutch size, and their relationship to the magnitude and direction of changes in PRL and LH. Earlier work within the normal range of clutch sizes found that increasing PRL between breeding attempts correlated with decreases in clutch size (Ryan et al. In prep). In the current

study we exposed latent plasticity by extending the phenotypic range of both clutch size and PRL using egg removal. However, this uncoupled rather than exaggerated any relationship between these two traits, inconsistent with a direct role for PRL in clutch size determination. We also found no evidence that individual variation in the magnitude and direction of changes in LH were associated with changes in PRL, as would be predicted by a systemic inhibitory effect of PRL on LH. Other studies have also not found evidence supporting an inhibitory effect of PRL on LH (Buntin et al. 1999; Small et al. 2007), including in breeding female zebra finches (Ryan et al. In prep). If changes in LH precede changes in PRL, or if both hormones are regulated independently (Goldsmith et al. 1984; Sharp et al. 1988), the central role of PRL in clutch size determination via inhibition of LH could be unfounded. Ultimately, we did not observe the individual correlations between PRL, LH and clutch size we would expect if these traits are linked mechanistically.

3.4.5. Coordination of incubation as an alternative hypothesis

Carefully designing our experiments around the time postulated to be invariantly linked to the inhibitory signal for clutch size determination in zebra finches (Haywood 1993c; Haywood 2013), we found no support for any relationship between circulating PRL and clutch size. This was true for Egg-removal females, as well as controls. Furthermore, individual changes in PRL were also not related to clutch size: PRL levels decreased between Pre-treatment and Treatment breeding attempts, but these changes were not associated with changes in clutch size, treatment or the response to treatment. If, contrary to the conclusions of Haywood (1993c), the inhibitory signal disrupting follicle growth varies temporally in zebra finches as it does in other species (Haywood 1993a), a relationship between PRL and clutch size might have been revealed in PRL on days 10 or 17, or in the rate of change in PRL between these days. We found no evidence for such a relationship. However, the broad temporal associations between PRL, incubation, and the cessation of laying still warrant explanation (Haftorn 1981; Williams 2012, chap. 5). In species like zebra finches, where hatching is more or less synchronous, incubation starts later for females laying larger clutches (Zann and Rossetto 1991). Though other factors play a role, females with eggs removed seem to initiate incubation later still (Gorman and Nager 2003). Since incubation can be delayed to the day the last or

second last egg is laid, and the time between follicle recruitment and laying is roughly four days (Haywood 1993c), females appear to be coordinating incubation with clutch *completion*, rather than clutch *size*. Under this scenario, females nearest to clutch completion *irrespective of clutch size* should show elevated PRL relative to those further away. Although not one of our original predictions, we examined PRL at the last measured time point prior to clutch completion to test this hypothesis. At the last measured time point prior to clutch completion for each female, PRL levels predicted the number of days to the cessation of laying, regardless of clutch size. Those females who were closest to completing their clutches had the highest PRL, whereas those who were relatively far from clutch completion had lower PRL. This was also true for the Pre-treatment breeding attempt, but only when including three females that laid particularly large clutches, specifically clutches in the range of those laid by Egg-removal females in the Treatment breeding.

3.4.6. Conclusions

Although the aim of the current paper was not to study the relationship between PRL and the temporal proximity to clutch completion, a role for PRL in the coordination of incubation with clutch completion in the absence of any effect on clutch size could explain equivocal support for the PRL-based mechanistic model (Millam et al. 1996; Sockman et al. 2000; Ryan et al. In prep). Since PRL measurements are generally taken early on or midway through laying, when clutch completion and development of full incubation is invariably closer for females laying smaller clutches, higher PRL could appear to correspond to fewer total eggs laid. In our study, this relationship was only observed in females laying naturally large (Pre-treatment) or experimentally-enlarged (Treatment) clutches, and may be explained by the timing of our blood sample. This is not to say that the rate of incubation onset necessarily dictates clutch size (Meijer et al. 1990), since the last follicle may have been ovulated at the time when PRL levels and time incubating begin their most rapid ascent. Rather, to the extent that variation in PRL levels reflect development of incubation behaviour, females could be coordinating two independently regulated processes, incubation and clutch completion, perhaps to minimize hatching asynchrony and sibling hierarchy (Sockman et al. 2006). Coordinating incubation and clutch completion to minimize sibling hierarchy would be most beneficial

to females laying the largest clutches, either naturally or 'artificially' through egg removal, as we observed in our study. Thus the link between incubation, PRL, egg laying, and clutch size appears complex. Tactile stimulation from the eggs is important in the cessation of laying, and has a stimulatory effect on PRL in many species, yet the variability in our responses to treatment demonstrate that it is not critical in the laying of a normal-sized clutch, nor in the rise in PRL during laying, i.e. some birds stopped laying even though eggs were removed, and these birds were not characterized by higher PRL levels than birds that continued laying. As shown by our data, PRL and clutch size can be largely uncoupled but there is a consistent rise in PRL with proximity to clutch completion. Although unlikely to be associated with the cessation of laying *per se*, this increase in PRL may reflect individually-variable strategies in development of incubation behaviour and hatching synchrony.

3.5. References

Angelier, Frederic, and Olivier Chastel. 2009. "Stress, prolactin and parental investment in birds: A review." *General and comparative endocrinology* 163 (1-2) (September): 142–148.

Angelier, Frédéric, Scott A. Shaffer, Henri Weimerskirch, and Olivier Chastel. 2006. "Effect of age, breeding experience and senescence on corticosterone and prolactin levels in a long-lived seabird: The wandering albatross." *General and Comparative Endocrinology* 149 (1) (October): 1–9. doi:10.1016/j.ygcen.2006.04.006.

Bailey, Robert E. 1950. "Inhibition with Prolactin of Light-Induced Gonad Increase in White-Crowned Sparrows." *The Condor* 52 (6) (November 1): 247–251. doi:10.2307/1364516.

Bentley, G.E., A.R. Goldsmith, A. Dawson, L.M. Glennie, R.T. Talbot, and P.J. Sharp. 1997. "Photorefractoriness in European Starlings (*Sturnus vulgaris*) Is Not Dependent upon the Long-Day-Induced Rise in Plasma Thyroxine." *General and Comparative Endocrinology* 107 (3) (September): 428–438. doi:10.1006/gcen.1997.6941.

Bolton, Mark, Pat Monaghan, and David C. Houston. 1993. "Proximate determination of clutch size in lesser black-backed gulls: the roles of food supply and body condition." *Can. J. Zool.* 71 (2): 273–279. doi:10.1139/z93-039.

Book, Cynthia M., J.R. Millam, Michael J. Guinan, and Ralph L. Kitchell. 1991. “Brood patch innervation and its role in the onset of incubation in the turkey hen.” *Physiology & Behaviour* 50 (2) (August): 281–285. doi:10.1016/0031-9384(91)90067-X.

Buntin, J.D., J.P. Advis, M.A. Ottinger, R.W. Lea, and P.J. Sharp. 1999. “An Analysis of Physiological Mechanisms Underlying the Antigonadotropic Action of Intracranial Prolactin in Ring Doves.” *General and Comparative Endocrinology* 114 (1) (April): 97–107. doi:10.1006/gcen.1998.7239.

Buntin, J.D., R.M. Hnasko, P.H. Zuzick, D.L. Valentine, and J.G. Scammell. 1996. “Changes in Bioactive Prolactin-like Activity in Plasma and Its Relationship to Incubation Behaviour in Breeding Ring Doves.” *General and Comparative Endocrinology* 102 (2) (May): 221–232. doi:10.1006/gcen.1996.0063.

Charmantier, Anne, Christopher Perrins, Robin H. McCleery, and Ben C. Sheldon. 2006. “Evolutionary Response to Selection on Clutch Size in a Long‐Term Study of the Mute Swan.” *The American Naturalist* 167 (3) (March 1): 453–465.

Charnov, Eric L., and John R. Krebs. 1974. “On clutch-size and fitness.” *Ibis* 116 (2): 217–219. doi:10.1111/j.1474-919X.1974.tb00241.x.

Decker, Karie, Courtney Conway, and Joseph Fontaine. 2012. “Nest predation, food, and female age explain seasonal declines in clutch size.” *Evolutionary Ecology* 26 (3): 683–699. doi:10.1007/s10682-011-9521-7.

Delehanty, David J., Lewis W. Oring, Albert J. Fivizzani, and Mohamed E. El Halawani. 1997. “Circulating Prolactin of Incubating Male Wilson’s Phalaropes Corresponds to Clutch Size and Environmental Stress.” *The Condor* 99 (2) (May 1): 397–405. doi:10.2307/1369946.

El Halawani, M. E., W. H. Burke, J. R. Millam, S. C. Fehrer, and B. M. Hargis. 1984. “Regulation of prolactin and its role in gallinaceous bird reproduction.” *Journal of Experimental Zoology* 232 (3) (December): 521–529. doi:10.1002/jez.1402320319.

Flint, Paul L., J. Barry Grand, Thomas F. Fondell, and Julie A. Morse. 2006. “Population Dynamics of Greater Scaup Breeding on the Yukon-Kuskokwim Delta, Alaska.” *Wildlife Monographs* 162 (1): 1–22. doi:10.2193/0084-0173(2006)162[1:PDOGSB]2.0.CO;2.

Fox, J., and Sanford Weisberg. 2011. *An R companion to applied Regression*. Second. Thousand Oaks CA: Sage. http://socserv.socsci.mcmaster.ca/jfox/Books/Companion.

Goldsmith, A. R., S. Burke, and J. M. Prosser. 1984. “Inverse changes in plasma prolactin and LH concentrations in female canaries after disruption and reinitiation of incubation.” *Journal of Endocrinology* 103 (2) (November 1): 251–256. doi:10.1677/joe.0.1030251.

Gorman, Helen E., and Ruedi G. Nager. 2003. “State-dependent incubation behaviour in the zebra finch.” *Animal Behaviour* 65 (4) (April): 745–754. doi:10.1006/anbe.2003.2120.

Haftorn, Svein. 1981. “Incubation during the Egg-Laying Period in Relation to Clutch-Size and Other Aspects of Reproduction in the Great Tit *Parus major*.” *Ornis Scandinavica* 12 (3) (December 1): 169–185. doi:10.2307/3676074.

Hall, Michael R., and Arthur R. Goldsmith. 1983. “Factors affecting prolactin secretion during breeding and incubation in the domestic duck (*Anas platyrhynchos*).” *General and Comparative Endocrinology* 49 (2) (February): 270–276. doi:10.1016/0016-6480(83)90144-2.

Hamel, Sandra, Steeve D. Côté, Jean-Michel Gaillard, and Marco Festa-Bianchet. 2009. “Individual variation in reproductive costs of reproduction: high-quality females always do better.” *Journal of Animal Ecology* 78 (1): 143–151. doi:10.1111/j.1365-2656.2008.01459.x.

Haywood, S. 1993a. “Sensory and Hormonal-Control of Clutch Size in Birds.” *Quarterly Review of Biology* 68 (1) (March): 33–60.

———. 2013. “Review of Physiological Adaptations for Breeding in Birds by T. D. Williams 2012, Princeton University Press, Princeton and Oxford.” *Ibis* 155: 218–219.

Haywood, Sacha. 1993b. “Role of extrinsic factors in the control of clutch-size in the Blue Tit *Parus caeruleus*.” *Ibis* 135 (1): 79–84. doi:10.1111/j.1474-919X.1993.tb02812.x.

———. 1993c. “Sensory Control of Clutch Size in the Zebra Finch (*Taeniopygia guttata*).” *The Auk* 110 (4) (October 1): 778–786.

Hector, J.A.L., and A.R. Goldsmith. 1985. “The role of prolactin during incubation: Comparative studies of three Diomedea albatrosses.” *General and Comparative Endocrinology* 60 (2) (November): 236–243. doi:10.1016/0016-6480(85)90319-3.

Hiatt, Eve S., Arthur R. Goldsmith, and Donald S. Farner. 1987. “Plasma Levels of Prolactin and Gonadotropins during the Reproductive Cycle of White-Crowned Sparrows (*Zonotrichia leucophrys*).” *The Auk* 104 (2) (April 1): 208–217.

Klomp, H. 1970. “The determination of clutch-size in birds: a review.” *Ardea*.

Lack, David. 1947. “The Significance of Clutch-size.” *Ibis* 89 (2): 302–352. doi:10.1111/j.1474-919X.1947.tb04155.x.

Lea, R. W., A. S. M. Dods, P. J. Sharp, and A. Chadwick. 1981. “The Possible Role of Prolactin in the Regulation of Nesting Behaviour and the Secretion of Luteinizing Hormone in Broody Bantams.” *Journal of Endocrinology* 91 (1) (October 1): 89–97. doi:10.1677/joe.0.0910089.

Lea, R.W, R.T Talbot, and P.J Sharp. 1991. “Passive immunization against chicken vasoactive intestinal polypeptide suppresses plasma prolactin and crop sac development in incubating ring doves.” *Hormones and Behaviour* 25 (3) (September): 283–294. doi:10.1016/0018-506X(91)90002-Y.

Lescroël, Amélie, Katie M. Dugger, Grant Ballard, and David G. Ainley. 2009. “Effects of individual quality, reproductive success and environmental variability on survival of a long-lived seabird.” *Journal of Animal Ecology* 78 (4): 798–806. doi:10.1111/j.1365-2656.2009.01542.x.

Lessells, C. M., and Peter T. Boag. 1987. “Unrepeatable Repeatabilities: A Common Mistake.” *The Auk* 104 (1) (January 1): 116–121. doi:10.2307/4087240.

Lewis, Robert A. 1975. “Reproductive biology of the white-crowned sparrow. II. Environmental control of reproductive and associated cycles.” *Condor*: 111–124.

Maney, D. L., S. J. Schoech, P. J. Sharp, and J. C. Wingfield. 1999. “Effects of vasoactive intestinal peptide on plasma prolactin in passerines.” *General and comparative endocrinology* 113 (3) (March): 323–330.

March, J. B., P. J. Sharp, P. W. Wilson, and H. M. Sang. 1994. “Effect of active immunization against recombinant-derived chicken prolactin fusion protein on the onset of broodiness and photoinduced egg laying in bantam hens.” *Journal of Reproduction and Fertility* 101 (1) (May 1): 227–233. doi:10.1530/jrf.0.1010227.

Martin, T. E, R. D. Bassar, S. K. Bassar, J. J. Fontaine, P. Lloyd, H. A. Mathewson, A. M. Niklison, and A. Chalfoun. 2006. “Life-history and ecological correlates of geographic variation in egg and clutch mass among passerine species.” *Evolution* 60 (2): 390–398.

Massaro, Melanie, Alvin N. Setiawan, and Lloyd S. Davis. 2007. “Effects of artificial eggs on prolactin secretion, steroid levels, brood patch development, incubation onset and clutch size in the yellow-eyed penguin (*Megadyptes antipodes*).” *General and Comparative Endocrinology* 151 (2) (April): 220–229. doi:10.1016/j.ygcen.2007.01.034.

McCleery, R.H., R.A. Pettifor, P. Armbruster, K. Meyer, B.C. Sheldon, and C. Perrins. 2004. “Components of Variance Underlying Fitness in a Natural Population of the Great Tit *Parus major*.” *The American Naturalist* 164 (3) (September 1): E62–E72.

Meier, Albert H. 1969. “Antigonadal effects of prolactin in the white-throated sparrow, *Zonotrichia albicollis*.” *General and Comparative Endocrinology* 13 (2) (October): 222–225. doi:10.1016/0016-6480(69)90243-3.

Meier, Albert H., and Jerry W. Dusseau. 1968. “Prolactin and the Photoperiodic Gonadal Response in Several Avian Species.” *Physiological Zoology* 41 (1) (January 1): 95–103. doi:10.2307/30158487.

Meijer, Theo, Serge Daan, and Michael Hall. 1990. “Family Planning in the Kestrel (*Falco tinnunculus*): The Proximate Control of Covariation of Laying Date and Clutch Size.” *Behaviour* 114 (1/4): 117–136.

Millam, J.R., B. Zhang, and M.E. El Halawani. 1996. “Egg Production of Cockatiels (*Nymphicus hollandicus*) Is Influenced by Number of Eggs in Nest after Incubation Begins.” *General and Comparative Endocrinology* 101 (2) (February): 205–210. doi:10.1006/gcen.1996.0022.

Miller, David A., Carol M. Vleck, and David L. Otis. 2009. “Individual variation in baseline and stress-induced corticosterone and prolactin levels predicts parental effort by nesting mourning doves.” *Hormones and behaviour* 56 (4) (October): 457–464.

Murton, R.K., and N.J. Westwood. 1977. *Avian breeding cycles*. Oxford [etc.]: Clarendon Press.

Nager, R. G, P. Monaghan, and D. C Houston. 2000. “Within-clutch trade-offs between the number and quality of eggs: experimental manipulations in gulls.” *Ecology* 81 (5): 1339–1350.

Nussey, D. H, A. J Wilson, and J. E Brommer. 2007. “The evolutionary ecology of individual phenotypic plasticity in wild populations.” *Journal of Evolutionary Biology* 20 (3) (May 1): 831–844. doi:10.1111/j.1420-9101.2007.01300.x.

O’Dwyer, T. W., W. A. Buttemer, D. M. Priddel, and J. A. Downing. 2006. “Prolactin, body condition and the cost of good parenting: an interyear study in a long-lived seabird, Gould’s Petrel (*Pterodroma leucoptera*).” *Functional Ecology* 20 (5): 806–811. doi:10.1111/j.1365-2435.2006.01168.x.

Pettifor, R. A., C. M. Perrins, and R. H. McCleery. 1988. “Individual optimization of clutch size in great tits.” *Nature* 336 (6195) (November 10): 160–162. doi:10.1038/336160a0.

Pinheiro, J, D Bates, S DebRoy, D Sarkar, and the R Development Core Team. 2011. “nlme: Linear and nonlinear mixed effects models.” *R package version 3.1-98*.

R Core Development Team. 2011. “R: A language and environment for statistical computing, reference index version 2.12.2.” *R Foundation for Statistical Computing, Vienna, Austria.*

Reddy, I. J., C. G. David, and S. S. Raju. 2007. “Effect of suppression of plasma prolactin on luteinizing hormone concentration, intersequence pause days and egg production in domestic hen.” *Domestic animal endocrinology* 33 (2) (August): 167–175.

Ricklefs, Robert E. 2010. “Parental Investment and Avian Reproductive Rate: Williams’s Principle Reconsidered.” *The American Naturalist* 175 (3) (March 1): 350–361.

Rockwell, R. F., C. S. Findlay, and F. Cooke. 1987. “Is there an Optimal Clutch Size in Snow Geese?” *The American Naturalist* 130 (6) (December 1): 839–863.

Rowe, L., D. Ludwig, and D. Schluter. 1994. “Time, condition, and the seasonal decline of avian clutch size.” *The American naturalist* 143 (4): 698–722.

Rozenboim, I., C. Tabibzadeh, J. L. Silsby, and M. E. el Halawani. 1993. “Effect of ovine prolactin administration on hypothalamic vasoactive intestinal peptide (VIP), gonadotropin releasing hormone I and II content, and anterior pituitary VIP receptors in laying turkey hens.” *Biology of Reproduction* 48 (6) (June 1): 1246–1250. doi:10.1095/biolreprod48.6.1246.

Schaper, Sonja V., Alistair Dawson, Peter J. Sharp, Samuel P. Caro, and Marcel E. Visser. 2012. “Individual variation in avian reproductive physiology does not reliably predict variation in laying date.” *General and Comparative Endocrinology* 179 (1) (October 1): 53–62. doi:10.1016/j.ygcen.2012.07.021.

Sharp, P. J., M. C. Macnamee, R. J. Sterling, R. W. Lea, and H. C. Pedersen. 1988. “Relationships between prolactin, LH and broody behaviour in bantam hens.” *Journal of Endocrinology* 118 (2) (August 1): 279–286. doi:10.1677/joe.0.1180279.

Sharp, Peter J., Alistair Dawson, and Robert W. Lea. 1998. “Control of luteinizing hormone and prolactin secretion in birds.” *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology* 119 (3) (June): 275–282. doi:10.1016/S0742-8413(98)00016-4.

Sharp, Pj, Ic Dunn, and Rt Talbot. 1987. “Sex-Differences in the LH Responses to Chicken LHRH-I and LHRH-II in the Domestic-Fowl.” *Journal of Endocrinology* 115 (2) (November): 323–331. doi:10.1677/joe.0.1150323.

Small, Thomas W., Peter J. Sharp, and Pierre Deviche. 2007. “Environmental regulation of the reproductive system in a flexibly breeding Sonoran Desert bird, the Rufous-winged Sparrow, *Aimophila carpalis*.” *Hormones and Behaviour* 51 (4) (April): 483–495. doi:10.1016/j.yhbeh.2007.01.004.

Sockman, K. W., H. Schwabl, and P. J. Sharp. 2000. “The role of prolactin in the regulation of clutch size and onset of incubation behaviour in the American kestrel.” *Hormones and behaviour* 38 (3) (November): 168–176.

Sockman, Keith W., Peter J. Sharp, and Hubert Schwabl. 2006. “Orchestration of avian reproductive effort: an integration of the ultimate and proximate bases for flexibility in clutch size, incubation behaviour, and yolk androgen deposition.” *Biological Reviews* 81 (04) (October): 629. doi:10.1017/S1464793106007147.

Travers, Marc, Michael Clinchy, Liana Zanette, Rudy Boonstra, and Tony D. Williams. 2010. “Indirect predator effects on clutch size and the cost of egg production.” *Ecology Letters* 13 (8): 980–988. doi:10.1111/j.1461-0248.2010.01488.x.

Vézina, François, John R. Speakman, and Tony D. Williams. 2006. “Individually variable energy management strategies in relation to energetic costs of egg production.” *Ecology* 87 (10) (October 1): 2447–2458. doi:10.1890/0012-9658(2006)87[2447:IVEMSI]2.0.CO;2.

Williams, George C. 1966. “Natural Selection, the Costs of Reproduction, and a Refinement of Lack’s Principle.” *The American Naturalist* 100 (916) (November 1): 687–690.

Williams, T. D. 1996. “Intra-and inter-individual variation in reproductive effort in captive-breeding zebra finches (Taeniopygia guttata).” *Canadian journal of zoology* 74 (1): 85–91.

Williams, T. D., and M. Miller. 2003. “Individual and resource-dependent variation in ability to lay supranormal clutches in response to egg removal.” *Auk* 120 (2) (April): 481–489.

Williams, Tony D. 2008. “Individual variation in endocrine systems: moving beyond the ‘tyranny of the Golden Mean’.” *Philosophical Transactions of the Royal Society B: Biological Sciences* 363 (1497) (May 12): 1687 –1698. doi:10.1098/rstb.2007.0003.

Williams, Tony D. 2012. *Physiological Adaptations for Breeding in Birds*. Princeton, NJ: Princeton University Press, August 5.

Wingfield, John C., Brian K. Follett, Kathleen S. Matt, and Donald S. Farner. 1980. “Effect of day length on plasma FSH and LH in castrated and intact white-crowned sparrows.” *General and Comparative Endocrinology* 42 (4) (December): 464–470. doi:10.1016/0016-6480(80)90212-9.

You, S., L. K. Foster, J. L. Silsby, M. E. El Halawani, and D. N. Foster. 1995. “Sequence analysis of the turkey LH β subunit and its regulation by gonadotrophin-releasing hormone and prolactin in cultured pituitary cells.” *Journal of Molecular Endocrinology* 14 (1) (February 1): 117–129. doi:10.1677/jme.0.0140117.

Zadworny, D., K. Shimada, H. Ishida, C. Sumi, and K. Sato. 1988. “Changes in plasma levels of prolactin and estradiol, nutrient intake, and time spent nesting during the incubation phase of broodiness in the Chabo hen (Japanese bantam).” *General and Comparative Endocrinology* 71 (3) (September): 406–412. doi:10.1016/0016-6480(88)90269-9.

Zanette, Liana Y, Aija F White, Marek C Allen, and Michael Clinchy. 2011. “Perceived Predation Risk Reduces the Number of Offspring Songbirds Produce per Year.” *Science* 334 (6061) (December 9): 1398–1401. doi:10.1126/science.1210908.

Zann, R.A. 1996. *The zebra finch: a synthesis of field and laboratory studies*. Oxford University Press Oxford:

Zann, R, and M Rossetto. 1991. “Zebra Finch Incubation: Brood Patch, Egg Temperature and Thermal Properties of the Nest.” *Emu* 91 (2) (January 1): 107–120.

Zuur, Alain F., Elena N. Ieno, and Chris S. Elphick. 2010. “A protocol for data exploration to avoid common statistical problems.” *Methods in Ecology and Evolution* 1 (1): 3–14. doi:10.1111/j.2041-210X.2009.00001.x.

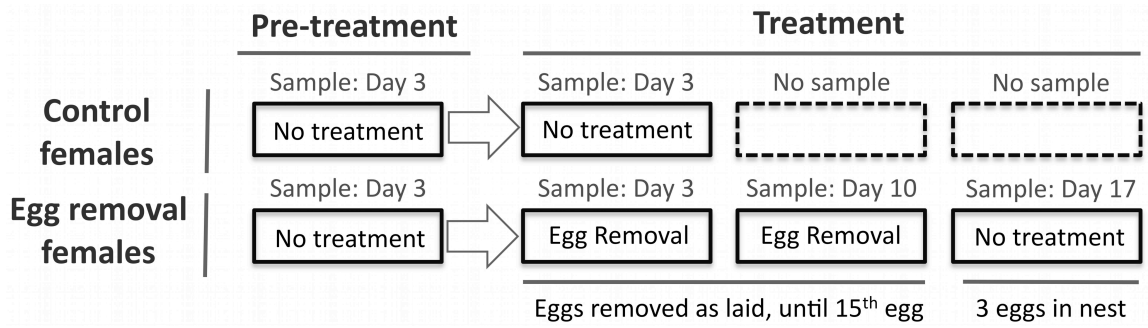

Figure 3.1. ***Flowchart illustrating experimental design and analytical framework for testing the PRL-based mechanistic model for avian clutch size determination in captive zebra finches using egg removal. Intra-individual comparisons for both control and egg removal females were made between Pre-treatment and Treatment clutch sizes and PRL at the putative time of follicular inhibition (egg day 3). Comparisons between day 3 PRL and final clutch size were also made both within and between treatment groups for the Treatment breeding. Finally, individual rate of change in PRL through the Treatment (days 3 to 10 and 10 to 17) and final clutch size were examined.***

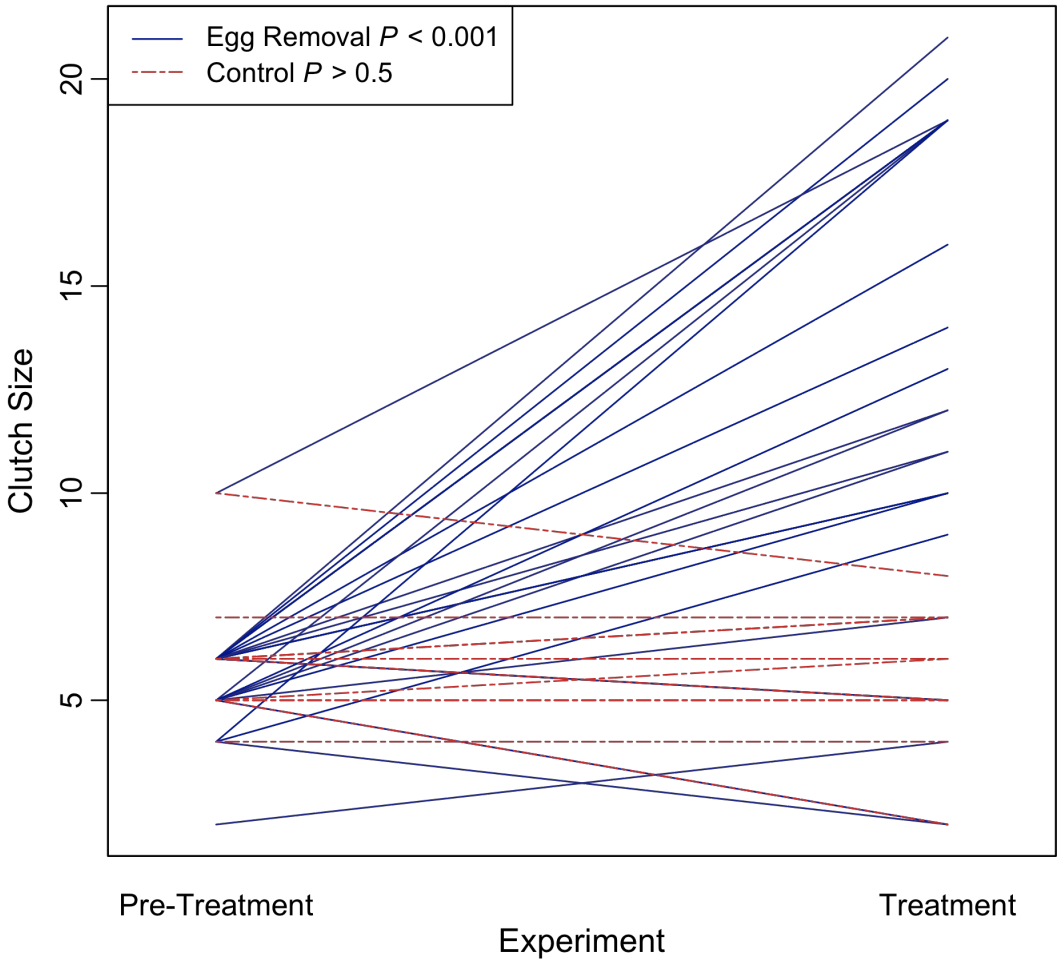

Figure 3.2. ***Individual changes in clutch size between Pre-treatment and Treatment breeding attempts for Egg-removal (solid blue lines) and Control (no egg removal; dashed red lines) female zebra finches.***

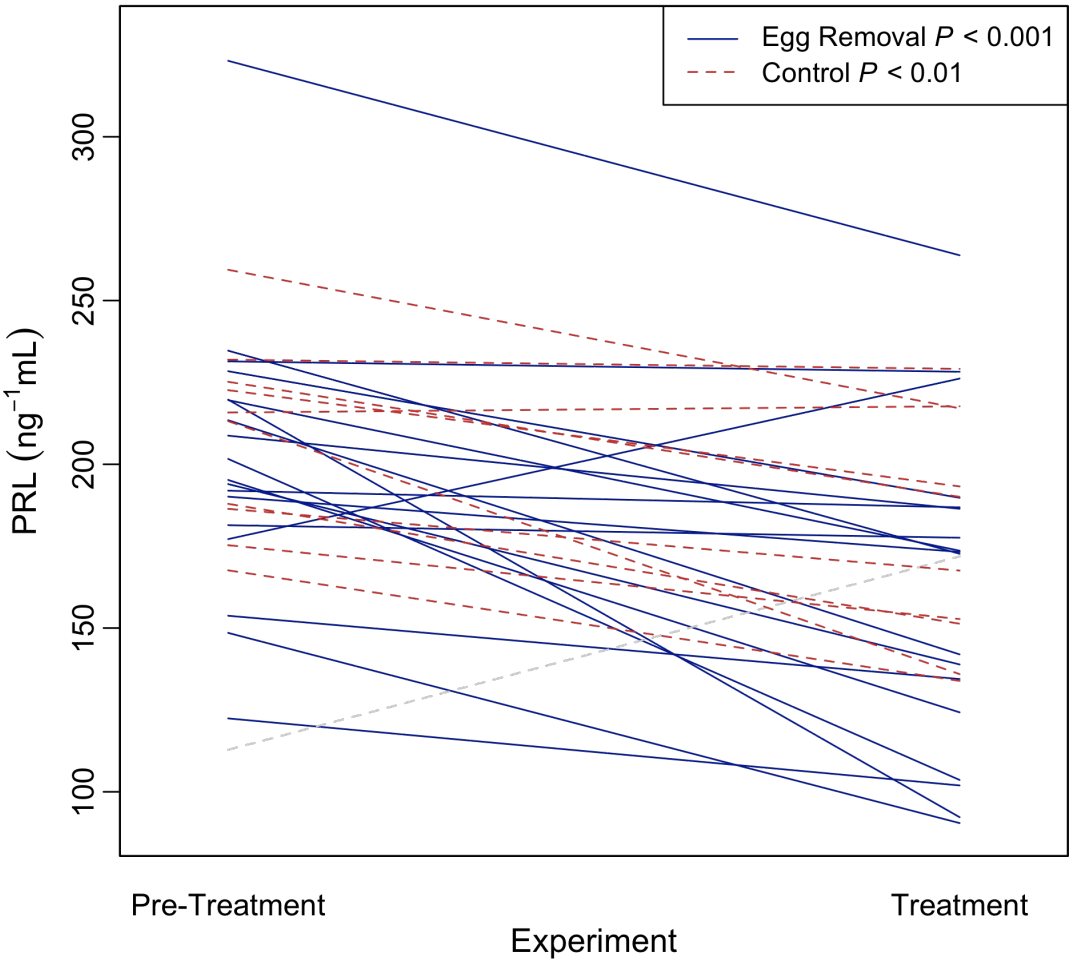

Figure 3.3. ***Individual changes in PRL between the Pre-treatment and Treatment breeding attempts for Egg-removal (solid blue lines) and Control (dashed red lines) female zebra finches, taken on the day the third egg was laid. P-values are based on the exclusion of data point with high leverage in the original model (shown as dashed grey line).***

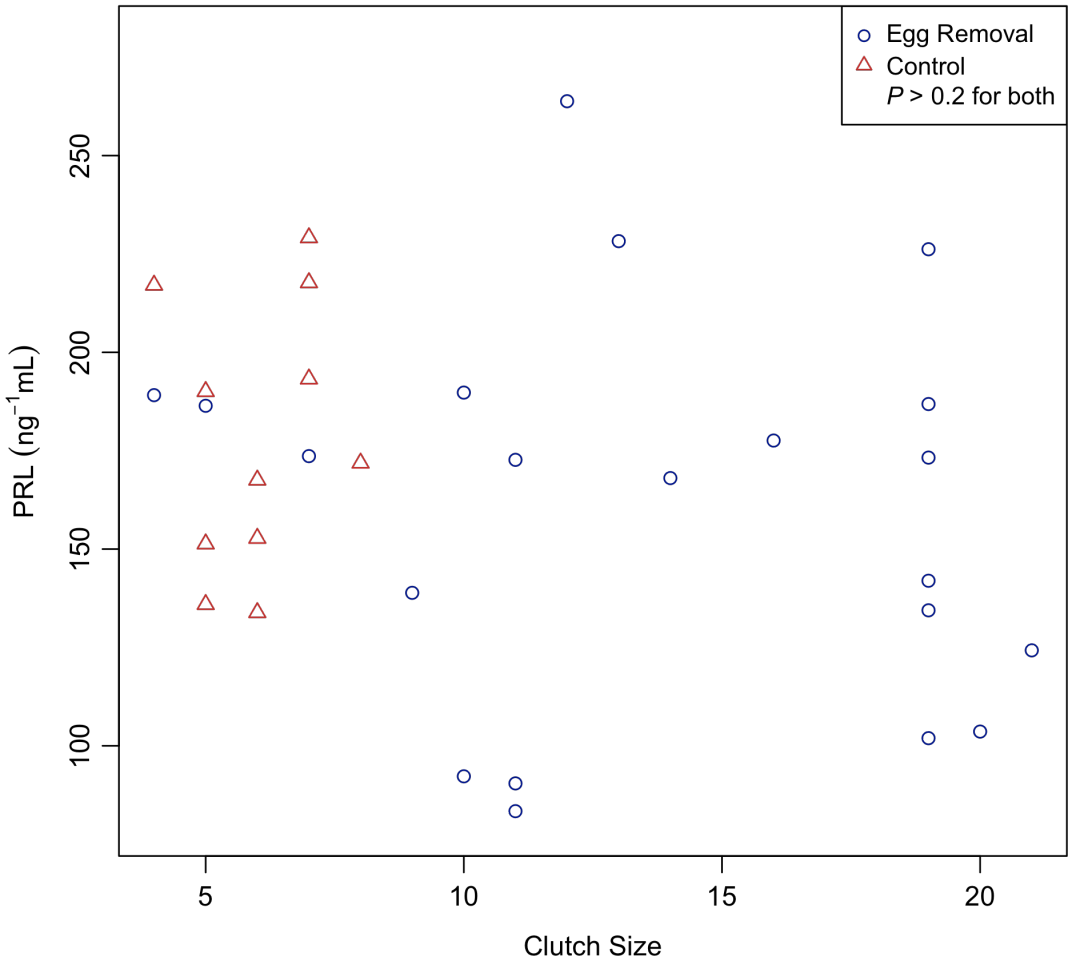

Figure 3.4. ***Plasma PRL on the day the third egg was laid during the Treatment breeding, for Egg-removal (blue circles) and Control (red triangles) female zebra finches. There were no significant relationships between the two variables for either group.***

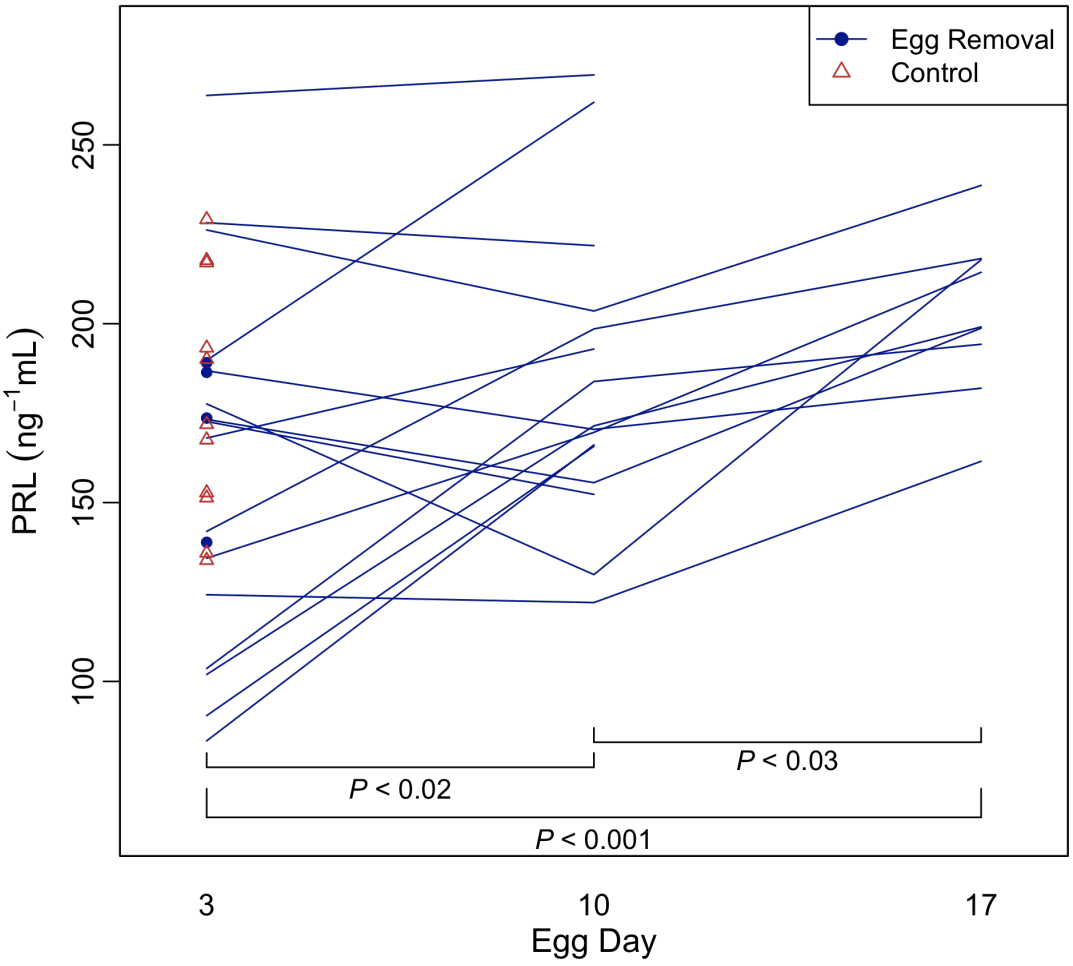

Figure 3.5. ***Plasma PRL by egg day for laying female zebra finches during the Treatment breeding. PRL for Egg-removal females laying 10 or more eggs are described by blue lines, whereas Egg-removal females laying fewer than 10 eggs are shown as solid blue circles. Control females, all of which laid less than 10 eggs are described with red triangles.***

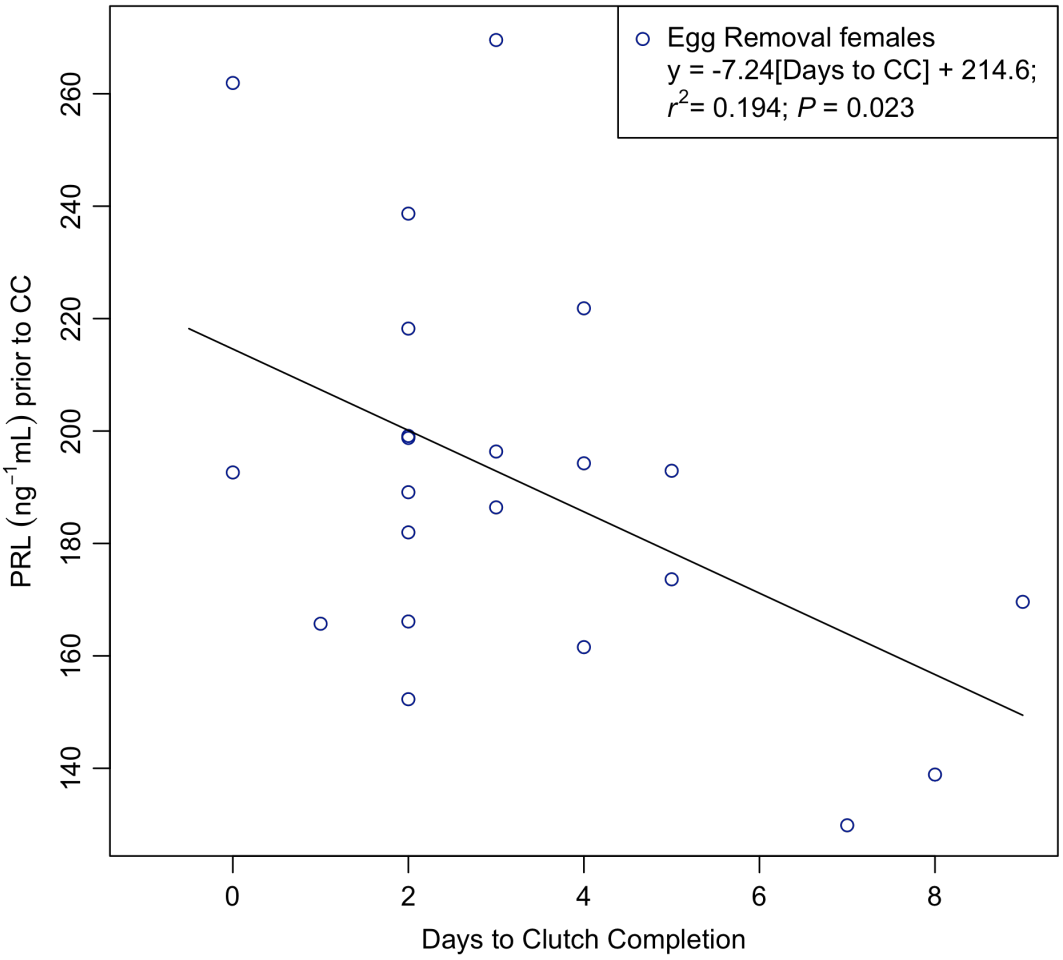

Figure 3.6. ***Plasma PRL for the blood sample closest to clutch completion and the number of days until the last egg laid for Egg-removal female zebra finches. Females closest to clutch completion at the time of hormone measurement had significantly higher PRL than those further away. A significant relationship between these two traits was also found for the Pre-treatment breeding.***

Chapter 4.

General Synthesis and Future Directions

4.1. Synthesis

The primary goal of this thesis was to experimentally test the mechanistic hypothesis that circulating PRL levels during laying act as the predominant regulatory factor in avian clutch size determination. I found no evidence for such a relationship in breeding zebra finches, nor did I find evidence that pre-breeding PRL levels predict subsequent clutch size. I also ultimately rejected the hypothesis that changes in PRL levels (i.e. plasticity) both within and between breeding attempts are linked to clutch size determination. Cumulatively, these findings do not support the PRL-based mechanistic model whereby circulating PRL either directly inhibits laying, or acts indirectly effect via inhibition of LH at the level pituitary. Nonetheless, a complete rejection of any role for PRL in clutch size determination would be premature, and alternative explanations for our findings are worth considering.

4.2. Other Considerations and Future Directions

We specifically designed the timing of our blood sampling for PRL measurement around the time when follicular inhibition in zebra finches is hypothesized to occur (Haywood 1993b). Based on a series of egg addition and removal experiments, Haywood (1993b; 2013) explicitly states that the inhibition of follicle growth in zebra finches occurs six hours (± 1 hour) after dawn on the day the third egg is laid, regardless of the size of clutch ultimately laid. All of our egg day 3 PRL values were taken within one hour of this temporal window, yet we found no evidence for an effect of PRL on clutch size at this time. Haywood (1993b) proposed a regulatory mechanism for clutch

size which operates via the disruption of follicle growth rather than the inhibition of pre-vitellogenic follicle recruitment. However, this hypothesis fails to explain how an invariably timed inhibitory signal might operate, e.g. how birds laying experimentally-enlarged clutches in response to egg removal subsequently cease laying when eggs are left in the nest. Furthermore, Haywood's conclusions are based on his exclusion of clutches outside the range of 4-6 eggs, despite the fact that zebra finches regularly lay 2-7 eggs in the wild, not to mention supra-normal clutches laid in response to egg removal in this study and others (Williams and Miller 2003). Thus the hypothesis that the timing of follicular inhibition is temporally invariable (Haywood 1993b) in zebra finches could in part be an artefact of this studies' data exclusion criteria. If this is true, the timing of follicular inhibition may vary in relation to timing of laying, as suggested for other species (Haywood 1993a). The possibility that the timing of follicular inhibition by PRL varies temporally was considered in chapter 3. Due to the size of zebra finches and the volume of plasma required for our assays, we were limited to sampling the same female no more often than once a week. However, if contrary to Haywood's suggestion the timing of follicular inhibition does vary, it is reasonable to conclude that we would have captured this effect in our analysis of the rate of increase between days 3 and 10, or day 10 and 17. For example, if follicular inhibition occurs on the day the 9th egg is laid for a female laying 11 eggs, but on the day the 12th egg is laid for a female laying 14 eggs, then presumably the female laying 11 eggs would exhibit a higher rate of PRL increase, or higher PRL on egg day 10, than the female who laid 14 eggs. We saw no evidence for such a relationship. As the time roughly corresponding to midway through laying, egg day 3 still represents a strategic time to look for an inhibitory effect of PRL, even if Haywood's claims prove false. Nonetheless, it would still be worthwhile to examine PRL levels at other times in laying zebra finches. To address this issue, I carried out subsequent experiments looking at plasma PRL concentrations on the days the 2nd and 4th eggs are laid in relation to clutch size. Circulating PRL during these times would bracket the time postulated by Haywood (1993b; 2013), and could provide additional confidence in our conclusions about the lack of a role for circulating PRL as the inhibitory signal in clutch size determination. The results of these assays were not available for inclusion in this thesis, but based on the findings I described above I predict there to be no relationship between plasma PRL on these days and clutch size.

While we were interested in testing the more general interpretation of the PRL-based mechanistic model, whereby *circulating* PRL is the primary agent through which follicular recruitment or development is inhibited, it is possible that PRL exerts localized rather than systemic regulatory control. Given the many pleiotropic effects of PRL (Bole-Feysot et al. 1998), a plausible alternative to systemic control of clutch size stems from local regulation of an inhibitory PRL signal, e.g. via differential PRL receptor expression in specific target tissues. Two sites where local regulation by differential regulation of the PRL receptor (PRL-R) could play a role in clutch size determination are in the brain and the ovary. In the brain, PRL-R is most highly expressed in the anterior pituitary and the hypothalamus, the latter thought to be the major target for the central regulation of PRL secretion (Zhou et al. 1996; Ohkubo et al. 1998a; Ohkubo et al. 1998b). Although PRL-R mRNA is more highly expressed in the pituitaries of incubating hens relative to laying hens (Ohkubo et al. 1998a), PRL-R expression in the hypothalamus does not differ between two strains of chicken with differing tendencies for broody behaviour, a behaviour itself associated with the cessation of laying (Ohkubo et al. 1998b). At least in some species, PRL-R expression in the pituitary and hypothalamus appear to be up- and down-regulated, respectively, by circulating PRL, as part of a self-regulatory feedback system (Zhou et al. 1996). If this is true in zebra finches, and PRL-R density in these regions somehow regulates clutch size determination, a relationship between PRL-R and clutch size should have been revealed indirectly with circulating PRL acting as a proxy for pituitary and hypothalamic PRL-R. We found no evidence for this in the form of circulating PRL, though the neuroendocrine mechanism for the regulation of plasma PRL could be more complex (Sharp and Blache 2003). Thus it is possible that pituitary and hypothalamic PRL-R expression plays a regulatory role in avian reproduction and possibly clutch size determination, though at present there is little evidence supporting this.

Another candidate tissue for the localized control of the effects of PRL via differential PRL-R expression is the ovary. In the ovary, a cellular pathway that regulates the process of recruitment of small white follicles into the hierarchy of yolky follicles has been proposed (Johnson and Woods 2009). This pathway is thought to involve an 'extrinsic' (i.e. endocrine) factor which regulates recruitment based on physiological and environmental signals (Johnson and Woods 2009). Whether or not this extrinsic signal

involves PRL is not clear, but several lines of evidence support some local regulation of follicular recruitment, and by extension, clutch size, by PRL at the level of the ovary. PRL-R is more highly expressed in the ovaries of laying compared to pre-laying birds (Kang et al. 2009; but see Zhou et al. 1996), and in higher concentrations in small white follicles compared to large yolky follicles (Zadworny et al. 2002). Furthermore, PRL inhibits growth of, and estrogen (E2) and progesterone (P4) production in, small white follicles (Zadworny et al. 1989; Hrabia et al. 2004). In contrast, PRL tends to stimulate E2 and P4 production in large yolky pre-ovulatory follicles (F1-F3), although this effect varies with dose and timing to ovulation (Hrabia et al. 2004). Indeed, steroidogenesis is an important component not only of follicular recruitment, but also of ovulation (Wilson and Sharp 1976; Johnson et al. 1985). This suggests that by feeding into the expression of follicle-stimulating hormone (FSH) and LH receptor pathways and steroidogenesis (see also Tabibzadeh et al. 1995), differential expression of PRL-R could overlay systemic changes in PRL, exerting control over clutch size locally in the ovary. One way to test this possibility would be to examine PRL-R expression differences in small white follicles that will or will not subsequently be recruited into the follicular hierarchy. While it can be difficult to determine which follicles will be recruited and later ovulated, removing eggs as we did in the second study could provide a way around this challenge. This approach would involve comparing small white follicles early in laying for control females and those whose eggs are removed, using previous breeding performance as a predictor of response and subsequent clutch size. PRL-R expression should be high for small white follicles belonging to females laying normal-sized clutches, but should remain low for egg removal females who will continue to lay. Large yolky follicles in the process of atresia would also be expected to exhibit higher PRL-R expression than yolky follicles proceeding to ovulation. Furthermore, PRL-R expression should be negatively correlated with E2 and P4 production by small white follicles, and positively correlated in large yellow follicles (Hrabia et al. 2004).

4.3. Conclusion

In conclusion, our experiments were designed to test a general interpretation of the PRL-based mechanistic hypothesis for clutch size determination, but our findings do not support the model in its current form. While alternatives hypotheses like those

described above may aid in the refinement of the PRL-based model, it remains possible that this proposed mechanism is based on little more than a series of broad temporal correlations between PRL, incubation, and the cessation of laying. If this is true, avian endocrinologists are currently left empty-handed as to a mechanism for clutch size determination. Because an understanding of the trade-offs and carry-over effects surrounding life history traits are built upon the understanding of those traits in isolation, progress in evolutionary endocrinology will be hindered without some appreciation of the mechanisms of fundamental traits like clutch size (Williams 2012). Given their fitness implications, understanding the mechanisms of major life history traits like clutch size are essential to predicting how organisms came to be adapted to their environments, and in turn, their capacity to respond to change.

4.4. References

Bole-Feysot, Christine, Vincent Goffin, Marc Edery, Nadine Binart, and Paul A. Kelly. 1998. “Prolactin (PRL) and Its Receptor: Actions, Signal Transduction Pathways and Phenotypes Observed in PRL Receptor Knockout Mice.” *Endocrine Reviews* 19 (3) (June 1): 225 –268. doi:10.1210/er.19.3.225.

Haywood, S. 2013. “Review of Physiological Adaptations for Breeding in Birds by T. D. Williams 2012, Princeton University Press, Princeton and Oxford.” *Ibis* 155: 218–219.

Haywood, Sacha. 1993a. “Role of extrinsic factors in the control of clutch-size in the Blue Tit *Parus caeruleus*.” *Ibis* 135 (1): 79–84. doi:10.1111/j.1474-919X.1993.tb02812.x.

———. 1993b. “Sensory Control of Clutch Size in the Zebra Finch (*Taeniopygia guttata*).” *The Auk* 110 (4) (October 1): 778–786.

Hrabia, A., H. Paczoska-Eliasiewicz, and J. Rzasa. 2004. “Effect of prolactin on estradiol and progesterone secretion by isolated chicken ovarian follicles.” *Folia Biologica, 52* 3 (4): 197–203.

Johnson, A.L., and Dori C. Woods. 2009. “Dynamics of avian ovarian follicle development: Cellular mechanisms of granulosa cell differentiation.” *General and Comparative Endocrinology* 163 (1-2) (September): 12–17. doi:10.1016/j.ygcen.2008.11.012.

Johnson, Patricia A., A.L. Johnson, and A. van Tienhoven. 1985. “Evidence for a positive feedback interaction between progesterone and luteinizing hormone in the induction of ovulation in the hen, *Gallus domesticus*.” *General and Comparative Endocrinology* 58 (3) (June): 478–485. doi:10.1016/0016-6480(85)90122-4.

Kang, B., J. R. Guo, H. M. Yang, R. J. Zhou, J. X. Liu, S. Z. Li, and C. Y. Dong. 2009. “Differential expression profiling of ovarian genes in prelaying and laying geese.” *Poultry Science* 88 (9) (September 1): 1975–1983. doi:10.3382/ps.2008-00519.

Ohkubo, T., M. Tanaka, K. Nakashima, and P.J. Sharp. 1998a. “Relationship between Prolactin Receptor mRNA in the Anterior Pituitary Gland and Hypothalamus and Reproductive State in Male and Female Bantams (*Gallus domesticus*).” *General and Comparative Endocrinology* 111 (2) (August): 167–176. doi:10.1006/gcen.1998.7099.

Ohkubo, T., M. Tanaka, K. Nakashima, R.T. Talbot, and P.J. Sharp. 1998b. “Prolactin Receptor Gene Expression in the Brain and Peripheral Tissues in Broody and Nonbroody Breeds of Domestic Hen.” *General and Comparative Endocrinology* 109 (1) (January): 60–68. doi:10.1006/gcen.1997.7008.

Sharp, Peter J., and Dominique Blache. 2003. “A neuroendocrine model for prolactin as the key mediator of seasonal breeding in birds under long- and short-day photoperiods. .” *Canadian Journal of Physiology & Pharmacology* 81 (4) (April): 350.

Tabibzadeh, C, I Rozenboim, J L Silsby, G R Pitts, D N Foster, and M el Halawani. 1995. “Modulation of ovarian cytochrome P450 17 alpha-hydroxylase and cytochrome aromatase messenger ribonucleic acid by prolactin in the domestic turkey.” *Biology of Reproduction* 52 (3) (March 1): 600 –608. doi:10.1095/biolreprod52.3.600.

Williams, T. D., and M. Miller. 2003. “Individual and resource-dependent variation in ability to lay supranormal clutches in response to egg removal.” *Auk* 120 (2) (April): 481–489.

Williams, Tony D. 2012. “Hormones, life-history, and phenotypic variation: Opportunities in evolutionary avian endocrinology.” *General and Comparative Endocrinology* (0). doi:10.1016/j.ygcen.2011.11.028. http://www.sciencedirect.com/science/article/pii/S0016648011004424.

Wilson, Susan C., and P. J. Sharp. 1976. “Induction of Luteinizing Hormone Release by Gonadal Steroids in the Ovariectomized Domestic Hen.” *Journal of Endocrinology* 71 (1) (October 1): 87–98. doi:10.1677/joe.0.0710087.

Zadworny, D, N Kansaku, G Bedecarrats, D Guemene, and U Kuhnlein. 2002. “Prolactin and its receptor in galliformes.” *Avian and Poultry Biology Reviews* 13 (3): 7. doi:10.3184/147020602783698566.

Zadworny, D., K. Shimada, H. Ishida, and K. Sato. 1989. “Gonadotropin-stimulated estradiol production in small ovarian follicles of the hen is suppressed by physiological concentrations of prolactin in vitro.” *General and Comparative Endocrinology* 74 (3) (June): 468–473. doi:10.1016/S0016-6480(89)80044-9.

Zhou, J. Feng, D. Zadworny, D. Guemene, and U. Kuhnlein. 1996. “Molecular cloning, tissue distribution, and expression of the prolactin receptor during various reproductive states in *Meleagris gallopavo*.” *Biology of reproduction* 55 (5): 1081–1090.