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Vocal Handedness: The Emergence of Lateralization at Fledging

Rudy Bellani

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VOCAL HANDEDNESS: THE EMERGENCE OF LATERALIZATION AT
FLEDGING

A Thesis Presented to the Faculty of
The Rockefeller University
in Partial Fulfillment of the Requirements for
the degree of Doctor of Philosophy

by

Rudy Bellani

June 2012

VOCAL HANDEDNESS: THE EMERGENCE OF LATERALIZATION AT FLEDGING

Rudy Bellani, Ph.D.

The Rockefeller University 2012

Adult waterslager canaries produce the majority of their song under the control of the left hemisphere / left syringeal half. Unilateral section of the left treacheosyringeal nerve results in near complete loss of song while right denervation affects only a subset of call elements. This strong asymmetry for the production of a learned vocalization is reminiscent of the left hemisphere control for language in humans and provides a potentially powerful animal model to study lateralized behaviors in the vertebrate brain.

Earlier work on another songbird, the European chaffinch, *Fringilla caerulea*, suggested that the onset of lateralized vocalizations occurred before song learning took place, but did not provide information about when and where this functional asymmetry first occurred. This “when” and “how” became the focus of my thesis.

In chapter 2, I describe the vocal ontogeny of begging calls in the canary, *Serinus canaria*. I describe the major call types, focusing primarily on two calls I named type A and type B. I show that the two calls are not interchangeable and are produced in different contexts as well as in different positions in the begging

bout. Moreover, I show that B calls first appear as modifications of A calls and are mechanically produced differently by the bird. Using call structure analysis, I suggest that B calls may provide location information to parents, a suggestion that is supported by my finding that the emergence of B calls coincides with the time when young canaries leave the nest, which is referred to as 'fledging.'

In chapter 3, I performed unilateral denervations across begging call ontogeny and found that late, but not early begging calls are asymmetrically produced. I further show that this onset of asymmetric contributions to the begging call by the syrinx emerges suddenly across one day -the day B calls appear and birds fledge. Call analysis revealed that both A and B calls are affected and thus, fascinatingly, A calls, which are little affected by syringeal denervation before P15, fall asymmetrically under the influence of the left syringeal half at P16. Track tracing experiments reveal that the descending motor pathway for food begging in canaries projects ipsilaterally, suggesting that the left / right differences in vocal control observed at the level of the syrinx, mirror left / right differences in control that occur at higher brain areas.

In chapter 4, I describe that food deprivation can shift the timing of fledging earlier. Furthermore, fledging earlier was accompanied by B call production and the onset of lateralized begging calls. The correlative emergence of these phenomena in stressed and unstressed canaries is striking and I propose that B calls serve a new communicative need that arises with leaving the

nest -namely, locatability. The emergence of vocal lateralization when the bird leaves the nest, regardless of age at which it occurs, is also curious and I posit that if lateralization of function is a way of compartmentalizing the complex world, then perhaps laterality arises when this world is first met.

For Lindsay.

Acknowledgments

This section is perhaps longer than customary but after writing 350 pages on canaries, writing 9 on the humans that were instrumental in making it happen still seems woefully insufficient.

I would like to thank Rockefeller University for being, in many ways, a place of another time. The housing, stipend, research funds, light course requirements, lack of bureaucracy, support of professors and the other innumerable but significant amenities and comforts that the university provides have collectively allowed me to pursue science the way it was a long time ago: a hobby of the privileged. I arrived into comfortable housing, was given research funds and so much intellectual freedom that I chose my field of study, the lab I would work in and the project I would pursue based solely on my interests. As such I would like to thank the vision of **Detlev Bronk** and the generosity and wisdom of **John D. Rockefeller Jr.** for beginning what is still, a graduate program unlike any other. This is a feature of the Rockefeller University that I have loved and enjoyed but I hope is never taken for granted. Regression to the mean is easy.

Still, I am here, beautifully stationed next to East River because of the wonderful friendships of **Susan Bertram, Cheryl Conrad, Robert Sapolsky, Bruce McEwen, and Russ Romeo.** **Susan Bertram** allowed me to enter her laboratory as a college freshman and though we eventually published a series of

papers, I think she soon realized she needed a more stringent screening process for incoming students. I once stupidly exterminated our cricket colony, essentially ending 6 months of our work, and in that low moment, she showered me with friendship and mentoring. That is how incredibly kind and supportive **Susan** is and over the years she has become a wonderful friend and I will always consider my years in her lab running after crickets in the Texas heat some of my favorite. If **Susan** made me love science, **Cheryl Conrad** made it my identity. **Cheryl's** lab is one of the most nurturing environments for young scientists and I was merely one of the ten or so students that she pushed into graduate school in my time there. I will forever be thankful for my years in her lab learning how to perform stereotaxic surgeries, running behavior, analyzing data, and becoming who I am and what I'm interested in scientifically. Thank you **Cheryl** for all the years of being an incredible magnet to science for so many of us. While in Cheryl's lab, I became enamored with **Robert Sapolsky's** writing and emailed him a series of science proposals which were eventually given the green light. **Cheryl** gave me all of her support and I spent two glorious months living my dream of being in **Robert's** lab running my own project. I want to thank **Robert** for the wonderful advice he gave me throughout my time there, for always responding to my naive emails on human evolution or the animal mind with great gusto even at 2 in the morning. I want to thank him for letting me sleep on his lab's couch for a month and a half because I didn't want to spend time commuting and for putting up with my hundred questions per lab meeting. While my project only kind of worked — agonizingly, so much more of science results in half-successes than complete

ones— the two months I spent in his lab were the intellectually freest of my life and I would like to thank **Robert** for them. Finally, I'd like to thank **Robert**, though he's never know it, for being the model of the kind of mind I strive, but fall so short, to be. A few months after returning to **Cheryl's** lab in Arizona, she pushed me to present my Honor's Thesis work to **Bruce McEwen's** lab during what was supposed to be a vacation in New York City. Simply remembering the one hour that I sat in the Weiss cafeteria thumbing my slides waiting to take the elevator ride to the 13th floor gives my adrenal glands a squeeze. I'd like to thank **Bruce** for being so wonderfully kind and having the manners of a prince when my presentation crashed his personal laptop and, when fixed, he sat through a silly overdressed undergrad giving him a twenty minute introduction to stress. Thank you **Bruce** for allowing me to present my work to your lab and afterwards for inviting me to join you a few months later during the summer. I spent three months in **Bruce's** lab and I spoke to him three times, twice in the bathroom, and yet that was all the convincing that I needed that the only place that I would apply to for graduate school was Rockefeller. I spent three months doing work with the supremely talented (and even funnier) **Russ Romeo** that resulted, along work that I did when I rotated through **Bruce's** lab, in the publication of 4 papers and a lifelong friendship. I was from the start biased to admire Russ because he loves science the way I do and it was inspiring to be around someone with so many interests that was so successful. It may seem strange but I want to thank **Russ** for allowing me to sleep under his bench in lab for two and a half months while Rockefeller found me housing for it allowed me to be exactly where I wanted to

be all the time. For the record, he DID numerously offer me his couch. Thanks for the awesome adventures in lab **Russ**.

Bruce McEwen has been incredibly supportive of me all of these years and I'd like to separately recognize what a wonderful mentor and inspiration he has been.

I'd like to thank **Cori Bargmann** for being on my thesis committee and providing outstanding leadership in the direction of the work presented here. **Cori**, because of her work ethic, sharpness of mind, and beautiful experimental approaches is an inspiration to many graduate students at Rockefeller and I am absolutely counted among those awed.

I'd like to thank Dr. **Sanford Braver** for teaching me everything I ever needed to know about statistics, namely, that they are not God. And that there is no excuse for sloppy or inappropriate tests. I have undoubtedly come up short somewhere in this thesis and the fault is all my own.

I joined the lab of Tao Sun at Cornell Medical School because of his molecular expertise and interest in brain asymmetry. Though we did not manage to utilize all of his molecular tools in the studies presented here, the time we spent together was some of my favorite while in graduate school. Tao managed to always push me to work harder while simultaneously cheering me on with

boundless energy. He is absolutely one of a kind and I have never been mentored by someone as enjoyable or encouraging as Tao. I only wish we could have played with manipulating genes a little bit more.

The past four years I have had a wonderful partner in **Fernando Nottebohm** and I could not thank him enough for providing me unmatched freedom during my thesis work. This freedom has at times been trying but has made me a better and much more mature scientist today than I could have been with a leash. **Fernando's** love of nature, and particularly his love of behavior have been inspirations to me and I only hope that my work presented here is some reflection of that. **Fernando** is, I hope he will not mind me saying so, an old-school scientist in the very greatest of ways. Beside being exacting, unwaveringly critical, forever skeptical and other traits that distinguish great scientific minds, he writes like a poet, distrusts new fads, is in science because he could not be anywhere else, and relishes, as he would say, tinkering. He is, if it is not obvious, a scientist after my own heart. **Fernando's** enthusiasm for discovery is unmatched and I sincerely hope that some of the discoveries made herein will arouse some of it and make him give that mischievous grin that so often has accompanied our conversations. Once, I commented to **Fernando** how I could crack egg shells, seal them with bird droppings, and the birds would hatch perfectly fine two weeks later. Considering the general lack of sterility in droppings, and that some birds construct nests utilizing them, it led us to wonder if in these droppings there might be some natural products that inhibited certain

types of bacterial growth. In other words, might there be antibiotics in bird poop? I never did chase this idea out, but these types of wonderful loose discussions were common and fueled the natural mischievous curiosity in me. The seemingly disparate chapter topics that occasionally occur herein are a reflection of all of that. Detlev Bronk once described the graduate fellow experience at Rockefeller thusly:

The University is not an aggregate of departments dealing with specialized fields of science. It is a community of scientific scholars who are free to follow their interests in any field of scholarship.

The students are few, and the faculty are many. This enables close association between the two, they live and work as junior and senior colleagues.

Students must be capable of self-directed study. Although many courses are offered, teaching is done primarily in seminars, in tutorial conferences, and in faculty research laboratories. There is thus considerable freedom for the active process of independent learning.

This was very much my experience here and I would like to thank **Fernando** for the friendship that accompanied an adventurous four years together.

The work that I did with **Rod Suthers** proved to be critical for the thesis presented and I'd like to thank him for letting me take residence in his lab for two and a half weeks. More than that, however, I'd like to thank him for the enthusiasm shown for the work we performed together and for taking me out for a beer when it wasn't working. The time I spent working alongside **Rod** reminded me of how much I love science and tinkering around with problems looking for solutions at a time when I felt discouraged, and I will forever be thankful for that.

Rod also became a friend and someone whose intellectual focus will stay with me as an unattainable but worthy aspirational goal -two results of my visit that do not show up in this thesis in figure form but are much more valuable to me.

Thank you **Rod. Amy Coy**, The Suther's lab laboratory assistant, made the entire trip possible by coordinating a million forms and was then wonderful and hilarious company while I was there.

I would like to thank the members of my laboratory that have helped me and accompanied me through this work. **Sattie Haripal**, the lab mom, has been a shining ray of happiness in the lab and is utterly one of a kind. The tissue-savant **Eben Pariser** helped me with some of the work presented here and I owe him many more beers than I ever gave him for all that assistance. Thank you very much **Eben. Wan-Chun Liu** is one of the most mischievous scientists I have ever met and I thank him for being a wonderful sounding board for my own quirky ideas. **Clare Walton** has been a great friend for many years and made my time at Rockefeller a much more fun one. **Scarlet Woodrick** was my summer student in 2010 and taught me how hard being a mentor to someone who works too efficiently, but worse, too fast, could be. Thank you for the magnificent help and all the laughs **Scarlet**. I want to thank **Amy Miller** for her patience (she certainly gave me a lot of it), for all the help, and for all the wonderful talk of food. I'm sorry we never made our own wheel of cheese while I was in the lab but I'm not yet ready to let the dream die. **Sharon Sepe** became a friend whose kindness knows no bounds. Separately, I would like to recognize **her** for all the effort she put into

the care of my canaries. I can not emphasize enough how important **Sharon** and the rest of the **Rockefeller Field Center staff** were to the work presented here. Wonderful animal care is critical for all studies dealing with behavior and none match the quality and attention to detail that my studies have benefited from at the **Field Center**. If only other labs could be so lucky.

During my time at Rockefeller I am also very proud to have created a summer neuroscience program for underrepresented high school students along with **Clare Walton** and would like to thank her for pouring her heart into the program and helping me to create such a wonderful environment for the students. I would like to thank **Fernando Nottebohm** for allowing us to visit the Rockefeller Field Research Center with our students -they loved every minute of it and **his** guided walks through the woods were often the highlight. I would like to thank the **Dean's Office**, and **Sid Strickland** in particular, for the support —our program would absolutely not have happened without it. I'd like to thank all of the mentors and teachers over the years that have helped our students experience the coolness of neuroscience including **Amani Ahmed, Lindsay Bellani, Nicole Bowles, Jake Brewer, Jenn Bussell, Ben Campbel, Christine Cho, Graeme Couture, Roman Corfas, Disan Davis, Moses Fester, Winrich Freiwald, Desmond Fugar, Jim Hudspeth, Ilia Karatsoreos, Andreas Keller, Katherine Leitch, Jeff Leitch, Wan-Chun Liu, Melinda Miller, Krystal Lozada, Sandy Simon, Russ Romeo, Brad Rosenberg, Bernice Rumala, Keith Tan, and Leslie Vosschal**. Finally, I'd like to thank **Paul Nurse, John Tooze, Rockefeller's**

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To my spectacular wife **Lindsay**, the woman I love and have promised to always love, to the woman that defines me and has given me a new life, thank you. Thank you for being so patient with me when this thesis took over our lives. Thank you for being my best friend and my laughing buddy, for being so loving to me when my behavior warrants so little to love, for enriching me and helping me through all 250 pages presented here. Jorge Luis Borges penned all of my feelings when he said, *being with you and not being with you is the only way I have to measure time*. He's good. And he's right.

I would like to thank my brother, **Rocky Canelas**, for being forever encouraging and for doing so with brave honesty, something I desperately need from time to time. This thesis was completed after a life lived alongside my brother and many of the best qualities of my work are derived from our friendship, from me learning to be his older brother, and from being taught that responsibility was from my younger one. Thank you Rock.

In closing, I would love to be able to say here -on record as it is- that I grew up a most studious young man but, in truth, I spent my youth in Bolivia riding the hell out of my bicycle as far and fast as I could before dinnertime. When the sun set and I was called home, dirty, sweaty, and happy, I ate dinner

and only *then* did I do something that anyone would now think was a premonition for where I would be today, sitting indoors facing a wall writing my Ph.D thesis while a beautiful day passes: I collapsed in bed, next to my bookshelf of a million books, and I read. And for that wonderful life that not so obviously but nevertheless absolutely pointed me here to Rockefeller, I'd like to thank my **mom.**

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

Peripheral asymmetries: human handedness.....	1
<i>Lateralized behaviors in the brain: human language.....</i>	<i>5</i>
<i>Anatomical asymmetries in the human brain.....</i>	<i>8</i>
The trouble with studying brain asymmetry.....	11
Overcoming the knowledge gap: model organisms.....	10
<i>The chicken and pigeon as models for the ontogeny of brain</i>	
<i>asymmetry.....</i>	<i>12</i>
<i>Brain asymmetry in songbirds.....</i>	<i>18</i>
<i>Sound production.....</i>	<i>19</i>
<i>Song development.....</i>	<i>23</i>
<i>The song system.....</i>	<i>25</i>
<i>The canary.....</i>	<i>31</i>
<i>The canary's song.....</i>	<i>34</i>
<i>Peripheral asymmetry in song production.....</i>	<i>34</i>
<i>Cerebral lateralization in song.....</i>	<i>38</i>
<i>Recent reinterpretations of early results on peripheral</i>	
<i>asymmetries in canaries.....</i>	<i>39</i>
Goals of this thesis.....	46

CHAPTER 2: FOOD BEGGING CALLS IN THE CANARY

Introduction.....	48
The ontogeny of begging calls, an introduction.....	51
Experiment 1: Are food begging calls similar across one day within hatchlings?.....	53
Experiment 2: Are begging calls significantly different between individuals?.....	61
Experiment 3: Can canary parents recognize their young by their A calls?.....	67
Experiment 4: How do begging calls change across development?.....	75
Experiment 5: Are begging calls sexually dimorphic?.....	86
Experiment 6: Are B calls a <i>structurally</i> distinct call type?.....	90
Experiment 7: Are B calls a <i>functionally</i> distinct call type?.....	96
Experiment 8: When do B calls appear in development?.....	103
Experiment 9: Why do B calls arise?.....	108
Experiment 10: Can B calls signal individuality?.....	113
Experiment 11: How do B calls arise?.....	116

Experiment 12: Are A and B vocalizations produced similarly?	124
Final thoughts.....	129

CHAPTER 3: EFFECTS OF DENERVATION

Introduction.....	133
Experiment 1: Are begging calls lateralized?.....	134
<i>Study 1A: Unilateral denervations in P7 - P8 canaries.....</i>	<i>134</i>
<i>Study 1B: Bilateral denervations in P8 canaries.....</i>	<i>138</i>
<i>Study 1C: Are begging calls lateralized in almost independent nestlings?</i>	<i>140</i>
Experiment 2: When and how do the asymmetric effects of denervation arise?	144
<i>Study 2A: When do left denervations wreck havoc?</i>	<i>144</i>
<i>Study 2B: Quantification of the effects of denervation.....</i>	<i>147</i>
<i>Study 2C: Are the effects of left denervation due to our surgery paradigm?</i>	<i>172</i>
Experiment 3: Are A or B calls preferentially affected?.....	174
Experiment 4: Do the calls recover following unilateral denervation?.....	180
Experiment 5: Is the syrinx innervated ipsilaterally or bilaterally?.....	183
Experiment 6: What is the nature of RA projections in the nestling?.....	194
Experiment 7: Is RA active in nestlings?	199

Final Thoughts	207
HOW CHAPTER 4 CAME TO BE	
What is so unique about being 16?.....	209
CHAPTER 4: HUNGER STRESS AND THE BEGGING CALL	
Experiment 1: What are the effects of hunger stress on P16 canaries?	211
Experiment 2: What are the effects of hunger stress in P14 canaries?	215
Experiment 3: What about the onset of fledging?	217
Experiment 4: And lateralized food begging calls?	220
Final thoughts	213
CHAPTER 5: CLOSING THOUGHTS	
Vocal ontogeny vs. The 3-stage model of song learning.....	225
The life history of our animals matters.....	228
The role of asymmetry.....	229
Future directions.....	230
A closing note.....	231
APPENDIX 1: GENERAL METHODS	
Software used	233
Subjects	233
Nestling Feeding	234
Perfusion of tissue	234
Sectioning of perfused tissue	236
Recording	236
Recording Preparation	236

Video Recording	237
Statistics and Graphing	237

APPENDIX 2: SOUND ANALYSIS TERMS

Call duration	239
Mean frequency	239
Pitch	239
Entropy	240
Frequency modulation	241

APPENDIX 3: PROTOCOLS

Cresyl Violet Stain.....	243
DNA purification.....	244
Sexing PCR.....	245
1:5 Nembutal	246
4% PFA.....	247
Cytochrome C Oxidase Staining	248

APPENDIX 4: BIRD SPECIES REFERENCED.....250

APPENDIX 5: IMAGE PROCESSING INFORMATION.....256

APPENDIX 6: ELECTROPORATION IN THE SONGBIRD BRAIN.....258

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

1.1: Schematic of how brain and peripheral asymmetries are currently understood to arise.....	7
1.2: The percent of all life-science papers published on brain asymmetry is increasing.....	14
1.3: The percent of brain-related papers investigating or mentioning brain asymmetry is increasing.....	15
1.4: The syrinx.....	20
1.5: The tracheosyringeal nerve.....	24
1.6: Vocal ontogeny and the currently accepted stages of song development.....	28
1.7: The oscine song system.....	29
1.8: Wild and domestic canary strains.....	33
1.9: Snippet of adult canary song.....	35
1.10: Left denervations of the tracheosyringeal nerve result in greater deficits than right denervations in adult canaries.....	37
1.11: Lesions of the left HVC result in greater deficits than lesions to the right HVC in adult canaries.....	43
1.12: Lesions of the left, but not right, HVC result in structural loss of the song and syllable stereotypy in outbred domestic canaries.....	45

CHAPTER 2: FOOD BEGGING CALLS IN THE CANARY

2.1: The ontogeny of begging calls of one individual.....	55
2.2: Food-begging calls differ between individuals but have similar components.....	57
2.3: Average pitch in two individuals decreases across development but there is great variability within any single day.....	59
2.4: The average pitch of food-begging calls within an individual can change dramatically from feeding to feeding.....	63
2.5: The characteristics of food-begging calls significantly differ between individual birds.....	65
2.6: Begging call discrimination assay.....	71
2.7: Female canaries preferentially respond to playback of their own young's begging calls.....	74
2.8: Average frequency of type A calls decreases across age.....	77
2.10: Average pitch of type A calls decreases across age.....	79
2.12: Average frequency modulation of type A calls increases across age.....	82
2.14: Average entropy of type A calls increases across age.....	84
2.16: Type A begging calls are sexually dimorphic in some but not all call features.....	88

2.17: Type A and B calls appear to be structurally distinct call types.....	91
2.18: A and B calls are structurally distinct call features.....	93
2.19: Type B calls appear to be produced at the beginning of begging bouts.....	95
2.20: Type B calls are predominantly produced at the beginning of a begging bout.....	105
2.21: Type B calls are first observed at P16.....	106
2.23: Fledging occurs largely at P16.....	110
2.24: B calls.....	111
2.25: The characteristics of B calls significantly differ between individual birds.....	115
2.26: Portions of early B calls visually resemble A calls.....	121
2.27: Portions of early B calls have similar call characteristics with A calls.....	123
2.28: Measurements of thoracic pressure and frequency of calls produced.....	127
2.29: Changes in pressure correlate strongly with changes of frequency in A calls but not B calls.....	131

CHAPTER 3: THE EMERGENCE OF LATERALIZATION IN FOOD-BEGGING CALLS

3.1: Unilateral denervations do not appreciably effect the structure of the earliest begging calls.....	137
3.2: Bilateral denervations in young canaries have no identifiable effects on begging calls.....	139
3.3: Left denervations late in begging ontogeny cause large changes to the begging call.....	141
3.4: Left denervations begin to cause large disorganizations in the structure of food-begging calls at P16.....	149
3.5: The effects of left P16 denervations across a begging bout.....	151
3.6: Left denervations have minimal effects on call structure at P15.....	153
3.7: Right denervations at P16 do not cause disorganizations in the structure of the food-begging call.....	155
3.9: At P16, the call characteristics of food-begging calls change significantly with left, but not right or sham, denervations.....	161
3.11: At P15, Neither left nor right denervation significantly change the call characteristics of food-begging calls.....	171
3.12: Denervation surgeries carried out on the same day produce similar results as overnight surgeries.....	175
3.13: Left denervation effects all calls.....	178

3.14: The structure of food-begging calls begins to deteriorate at P16 in animals that were denervated at P14 and this effect remains.....	185
3.15: The effects of left-denervation appear at P16 and are long lasting.....	186
3.16: Schematic of Dil and PRV injections.....	188
3.17: Each side of the syrinx is innervated by the ipsilateral n12.....	193
3.18: Injection of anterograde tracer into the left nucleus RA of adult canaries results in strong labeling of the left, but not right, n12ts.....	197
3.19: Nucleus RA projects to the ipsilateral n12 as early as P13.....	201
3.20: In P15 - P17 canaries, neither nucleus RA nor HVC is positive for cytochrome oxidase staining, but n12 and RA _m are.....	205
3.21: Quantitative densitometry of cytochrome oxidase staining in various brain nuclei.....	206

CHAPTER 4: HUNGER STRESS AND THE BEGGING CALL

4.1: In P16 birds, an 8hr bout of hunger stress induces greater B call production and this elevated production remains.....	214
4.2: In P14 birds, an 8hr bout of hunger stress induces the production of B calls and their production remains.....	216
4.3: An 8hr bout of hunger stress induces fledging in P14 canaries.....	219
4.4: Food deprivation stress induces lateralization of begging call.....	221

LIST OF TABLES

CHAPTER 2: FOOD BEGGING CALLS IN THE CANARY

2.9: Statistical comparisons of mean A call frequency from P9 - P21.....	78
2.11: Statistical comparisons of average A call pitch from P9 - P21.....	80
2.13: Statistical comparisons of average A call frequency modulation from P9 -P21.....	83
2.15: Statistical comparisons of average A call entropy from P9 - P21.....	85
2.22: Statistical comparisons of percent B calls produced at each age.....	107

CHAPTER 3: THE EMERGENCE OF LATERALIZATION IN FOOD-BEGGING CALLS

3.8: The changes to call characteristics following denervation at P16.....	158
3.10: The changes to call characteristics following denervation at P15.....	168

Definition of Terms and Abbreviations Used

Begging bout: is defined as the vocalizations produced between small feedings within one feeding session.

Feeding session: is defined as the feedings that occur at one time point until the bird is satiated. A feeding session is composed of multiple begging bouts as it takes multiple small drops of food to satiate a hatchling.

SAP: Sound Analysis Pro, a program built by Ofer Tchernichovski, Professor, Hunter College, to record, visualize, and analyze sound files with functions aimed at songbird researchers. Two versions were used throughout my studies, SAP V2.02 and SAP 2011. The version used for a particular analysis is noted where appropriate.

Fledging: The act of leaving the nest by birds. The onset of fledging is marked by postural changes that include perching while at rest or during feeding.

Peripheral asymmetry: Asymmetries in the use of bilateral structures outside of the brain. For example in the limbs for handedness or footedness, or in the vocal apparatus with vocal dominance in songbirds.

Neuroanatomical asymmetry: Asymmetries between the left and right central nervous systems at the level of anatomy. For example, the size of nuclei between the left and right cerebral hemispheres in humans. Asymmetries in anatomy may or may not lead to peripheral asymmetries.

Behavioral lateralization: When a behavior is asymmetrically represented between the two sides of the brain. This lateralization may be complete -wholly the dominion of one side- or only moderately asymmetric. This may or may not be the result of asymmetric anatomy and may or may not result in peripheral asymmetries.

Chapter 1: Introduction

Peripheral asymmetries: human handedness

The existence of handedness, the preference of one hand in the manipulation of objects or interaction with the environment, in the *Homo* taxon has been inferred from fossil remains as early as the middle (425,000 - 180,000 YBP) and early upper Pleistocene (180,000 - 10,000 YBP). Scratches on the labial surface of fossil incisors and canines suggest that *Homo neanderthalis* preferentially used one hand to hold a morsel (of meat, for example) between its teeth while using the other hand to cut it (Bermudez de Castro, Bromage, & Fernandez Jalvo, 1988; Lalueza & Frayer, 1997). Closer to modern day humans (though read: Burbano et al., 2010; Green et al., 2010), the existence of handedness in *Homo sapiens* has been described from studies investigating stone or wood artifacts, wear-marks on spoons and negative hand paintings in caves found in France and Spain (Bocquet, 1978; Conrnford, 1986; Groenen, 1997; Keeley, 1977; Rugg & Mullane, 2001; for review, see: Steele & Uomini, 2005; Westergaard & Suomi, 1996). Handedness has thus likely been present in humans since our earliest days, and as a result human customs throughout the world reveal a long history of left-right hand symbolism. For example, the right is symbolically synonymous with goodness and cleanliness in the majority of world cultures, from the Purum, a small tribe in the Indo-Burman border, to the Gogo people of Tanzania (For review, see: C. McManus, 2002). Many Middle-Eastern

and Asian cultures preserve the use of the right hand for eating and touching of the body above the waist and the left for cleaning, including after going to the bathroom, and the handling of genitals. The Bible, a text of significant influence in modern European, North and South American cultures, is full of left-right references that follow similar symbolic properties, as when speaking metaphorically of the Last Judgement:

³³ He will put the sheep on his right and the goats on his left. ³⁴ “Then the King will say to those on his right, ‘Come, you who are blessed ... ³⁵ For I was hungry and you gave me something to eat, I was thirsty and you gave me something to drink, I was a stranger and you invited me in, ³⁶ I needed clothes and you clothed me, I was sick and you looked after me, I was in prison and you came to visit me.’ ... ⁴¹ “Then he will say to those on his left, ‘Depart from me, you who are cursed ... ⁴² For I was hungry and you gave me nothing to eat, I was thirsty and you gave me nothing to drink, ⁴³ I was a stranger and you did not invite me in, I needed clothes and you did not clothe me, I was sick and in prison and you did not look after me.’ ... ⁴⁶ “Then they will go away to eternal punishment, but the righteous to eternal life.”

(Matthew 25, NIV)

However, it is not just pre-modern or religious cultures that morally differentiate the left and right hand. We use the word ‘right’ as a synonym for correct. We, today, shake hands and take oaths with the right hand. We call our closest most trusted confidant our ‘right hand man’, and we seat honored guests on our right side.

Handedness has been consciously appreciated for centuries¹. Yet even with such a long human history of acknowledging our handed asymmetries, the origins of how we came to express handedness remain obscure. Writing in the fourth century B.C.E. on whether handedness was innate or learned, Plato made his case thusly:

It is due to the folly of nurses and mothers that we have all become limping, so to say, in our hands. For in natural ability the two limbs are almost equally balanced; but we ourselves by habitually using them in a wrong way have made them different.

(Laws, Book VII)

A point for environment. Yet half a century later, Aristotle, who can arguably be credited with being the first modern developmental biologist, wrote oppositely:

For instance if we all constantly practiced throwing with our left hands, we should all become ambidextrous; yet the left hand is such by nature, and the right hand none the less superior to the left, however much we equalize the use of the two. Change of use does not abolish the natural distinction.

These arguments continued until the last few decades of scientific research.

Now, it has become clear that while societal factors can affect handedness

(Berdel Martin & Barbosa Freitas, 2003; Bryden, Ardila, & Ardila, 1993; Dellatolas et al., 1988), genetics undoubtedly play a role (Francks et al., 2003; Geschwind,

¹ Shakespeare makes 14 references to the “right hand” in all of his collected works, 2 of those are in reference to directionality such as “turn up on your right hand at the next turning” (Merchant of Venice: II, ii), while 6 speak of an action being taken by the right hand: “This poor right hand of mine is left to tyrannize upon my breast...then thus i thump it down” (Titus Andronicus: III, ii). Oppositely, he writes of the “left hand” only 4 times, 3 of which reference directions or location and the remaining instance has the hand sitting idly, though notably Shakespeare writes it to be burning “like twenty torches join’d” (Julius Ceasar: V, i).

Miller, DeCarli, & Carmelli, 2002; Klar, 2003, 2005; I. C. McManus, 1991; Ogdie et al., 2003; Sicotte, Woods, & Mazziotta, 1999; Warren, Stern, Duggirala, Dyer, & Almasy, 2006). For example, monozygotic twins, but not dizygotic twins, have higher handedness concordance rates than is expected by chance (Sicotte et al., 1999). Most convincingly, an ultrasound study identified 75 fetuses that were observed sucking their thumb in the womb and assessed their handedness at 10-12 years of age. The authors found that womb hand-preference for thumb-sucking predicted handedness in 70 of the children (93%) (Hepper, Wells, & Lynch, 2005). Thus, as fetuses lie outside of social influence, this is likely the best evidence yet that handedness has strong developmental underpinnings (Figure 1.1). Interestingly, all 5 of the miscategorized children were in the left thumb preference as a fetus group, suggesting that perhaps hand preference changed due to societal pressure. Alternatively, left-handedness may have a different developmental ontogeny than right-handedness and may not simply be the '*situs inversus*' of hand preference. Either way, how this behavior arises, when it does so, and where in the brain hand preference is encoded are all almost completely unknown. This however, might all be besides the point: though hand preference was the most widely discussed behavioral asymmetry in humans for centuries, the focus change in 1865.

Lateralized behaviors in the brain: human language

Dr. Paul Broca, a French physician working in Paris, attended to an epileptic patient named Leborgne (though tellingly nicknamed 'Tan' for the single word he produced) with severe language production problems. After Tan's sudden death in April of 1861, his brain was removed and the damage found was curiously very localized to the left frontal lobe. This curiosity may have remained so had happenstance not brought a similar patient in October of that year. Lélou, who suffered a stroke that rendered him unable to speak, was under the care of Dr. Broca when he too died, and his brain was similarly found to have specific damage to the same region of the left hemisphere. What Paul Broca argued later, after studying many more similarly affected patients, was that language was produced by the left hemisphere. As he put it:

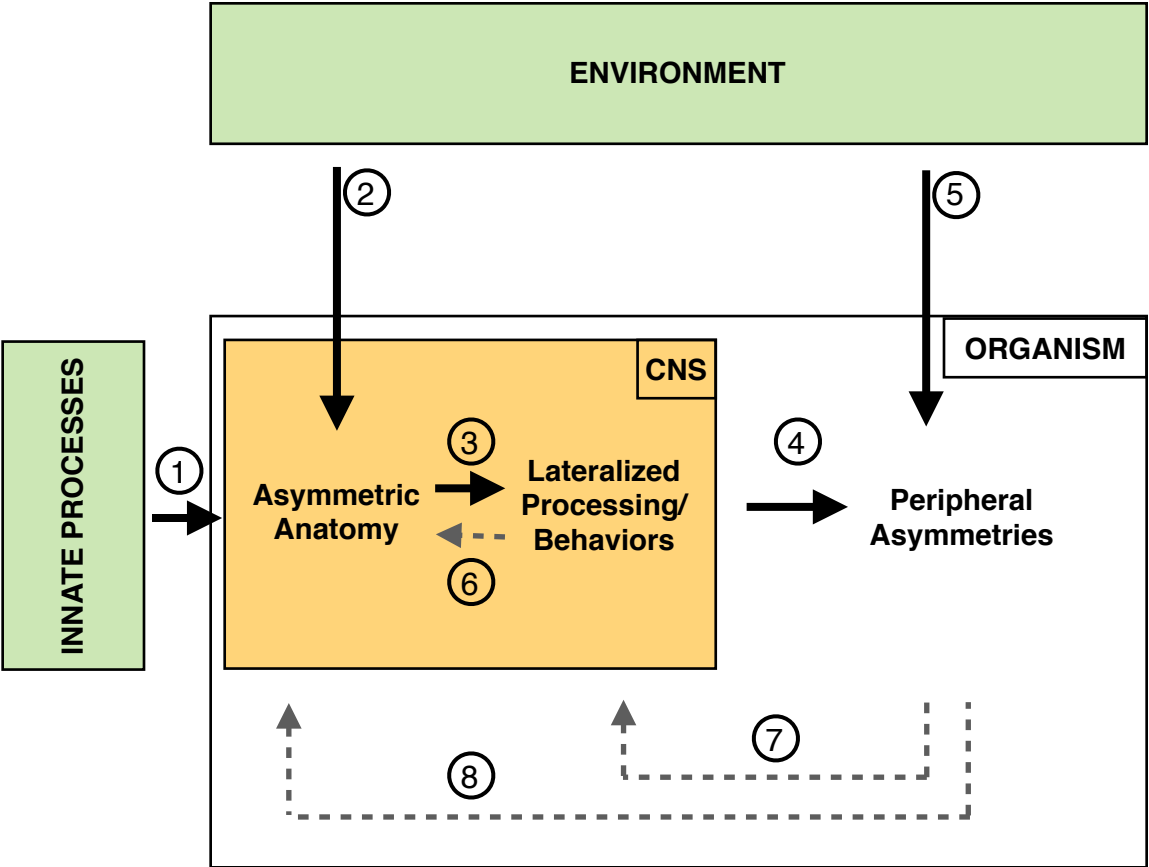
It would necessarily follow that the two halves of the brain do not have the same attributes - quite a revolution in the physiology of nervous centres. I must say that I could not easily resign myself to accept such a subversive consequence.

He was right to feel apprehensive, because it was indeed a revolution. And Paris could not stop talking about it. As Dr. Water Moxon wrote in 1866:

It is, I think, not over venturesome to say, that no observations have for many years excited in the medical world more intense and general interest as those of M. Broca.

Figure 1.1: Schematic of how brain and peripheral asymmetries are currently understood to arise. Asymmetric anatomy, arising from biological (1) or environmental influences (2) give rise to processes or behaviors being lateralized to one hemisphere over another (3), which can manifest in peripheral asymmetries (4) such as handedness in humans or eye preference in visual discrimination tasks in chicks or pigeons. These processes are thought to feedback on themselves (6, 7, 8) to reinforce and refine neural circuits, though experimental evidence for these mechanisms as they pertain to brain asymmetry is not well described. Environmental influence, say in the form of social customs, can also cause individuals to express peripheral asymmetries (4) that can then reorganize circuits (7, 8). Thus, for example, developmental processes may cause a child to be left-handed, but through training they may eventually be right-hand dominant. Solid arrows represent experimentally well-supported influences, while hashed arrows represent not well established influences or those still theoretical.

Figure 1.1



We now know that for the majority of humans, the left inferior frontal gyrus, now known as Broca's area, is dominant in the production and comprehension of language (Mohr et al., 1978). Indeed, 97% of right handers have left hemisphere dominance for language, while the remaining 3% have either right-sided or bilateral dominance. This relationship is not reversed in left-handers: 70% of left-handers are nonetheless left-hemisphere dominant for language, while 30% show right or bilateral activation (Branche, Milner, & Rasmussen, 1964; Coren, 1992). Thus, although handed preference can partially predict the side of hemispheric language localization, left-sided dominance for language is overwhelmingly represented in humans.

Anatomical asymmetries in the human brain

That behaviors such as language can be the dominion of one hemisphere over another launched work for the next 150 years aimed at uncovering underlying anatomical asymmetries that might explain lateralized behaviors in an otherwise seemingly symmetrical organ. Most obviously, scientists began to look at Broca's area, and numerous studies comparing the inferior frontal gyrus between the two hemispheres have shown that it is larger on the left than the right (Amunts et al., 1999; Falzi, Perrone, & Vignolo, 1982). Importantly, right-hemisphere speakers have reversed Broca's area asymmetry, with their right having more grey matter than their left (Dorsaint-Pierre et al., 2006). This finding

suggests a strong structure-function relationship, where the dominant language hemisphere has marked asymmetries neuroanatomically relative to the other (Figure 1.1).

Structurally, ultrasound studies have identified asymmetries between the left and right hemispheres as early as 20-22 weeks post fertilization (Hering-Hanit, Achiron, Lipitz, & Achiron, 2001), likely arising from early developmental programs. Indeed, gene expression asymmetries between the left and right hemispheres in future language producing areas can be identified as early as 12 weeks post-fertilization (Sun et al., 2005). In animal models of brain asymmetry, the earliest identified genes that guide the development of morphological left-right patterning in the central nervous system occur very early in development in both vertebrates (Signore et al., 2009) and invertebrates (Bauer Huang et al., 2007; Chuang, Vanhoven, Fetter, Verselis, & Bargmann, 2007), further strengthening the notion that cerebral asymmetries are guided, at least in part, by defined developmental programs. The left hemisphere is larger than the right in fetuses (Hering-Hanit et al., 2001) and neonates (Gilmore et al., 2007), but reverses by adulthood (Gur et al., 1999). Additionally, cytoarchitecturally, Broca's area, made up by Broadmann's area 44 and 45, does not develop adult-like asymmetries until 11 years and 5 years of age, respectively (Amunts, Schleicher, Ditterich, & Zilles, 2003). These slow maturing asymmetries may reflect natural developmental processes and/or behavioral reinforcement in the form, for example, of language practice. Indeed, individuals that incur left-hemisphere

damage overlying language areas early in life (<5 years of age) have stronger lateralization reversals (right-dominance) than those that acquire damage later in life (> 20 years of age; Muller et al., 1999), suggesting that there is ample experience-dependent plasticity in language regions during the first few years of life. Children with post-traumatic stress disorder lack frontal-lobe asymmetry (Carrion et al., 2001), further demonstrating that postnatal experience effects gross morphological asymmetries and potentially the development of the lateralization of behavior. Nevertheless, how genetics guide the establishment of morphological asymmetries in the vertebrate brain has been best described, although still not fully understood, in the midbrain of zebrafish (Barth et al., 2005; Bianco, Carl, Russell, Clarke, & Wilson, 2008; Carl et al., 2007; Inbal, Kim, Shin, & Solnica-Krezel, 2007; Miyasaka et al., 2009; Regan, Concha, Roussigne, Russell, & Wilson, 2009; Signore et al., 2009), following a long tradition of midbrain asymmetries described in amphibians and reptiles (Bisazza, Rogers, & Vallortigara, 1998; Concha & Wilson, 2001). However, we know very little about the genetics of left-right patterning in the telencephalon or, critically for the interests of this thesis, about when or how behaviors naturally become lateralized to one hemisphere over another. The reason for this dearth in understanding of asymmetry and lateralization mechanisms in vertebrates is primarily due to the lack of a good animal model system in which to study these phenomena.

The trouble with studying brain asymmetry

Brain asymmetry is still largely conceptualized as a feature of *some* neural systems in *some* —often “higher”— organisms. The reason for this is that symmetry/asymmetry has only been well mapped in primate and *C. elegans* nervous systems and is thus not often appreciated in many other model organisms. The nervous system of *C. elegans* has been exquisitely mapped all the way down to the origin of every cell (Kimble & Hirsh, 1979; Sulston & Horvitz, 1977). The human brain has also been well-mapped in terms of gross neuroanatomy due to centuries of work (LeMay, 1976; C. McManus, 2002; Swanson, 1995) but most recently due to CAT and MRI scan technologies which, because of the tracing or subtraction algorithms used to measure region morphology or their activity, discover asymmetries readily (For review, Toga & Thompson, 2003)². For the vast majority of other vertebrate model organisms however, there is no comparable understanding. This continues to be the case as a result of three principal factors: 1) The vast majority of neuroscience studies do not look for possible differences between hemispheres, 2) Our understanding of CNS development left to right in any vertebrate species is almost non-existent, and thus there are few described anatomical asymmetries to study, 3) Anatomical asymmetries can be slight or unexpectedly complex (Kawakami et al., 2003; Shinohara et al., 2008), both of which make them difficult to study. Furthermore,

² Of course, some of the asymmetries identified are pure artifact simply due to the multitude of voxel-based comparisons, even if proper statistical procedures account for alpha inflation. Still, it is noteworthy that a great majority of papers utilizing these technologies find differences between the left and right anatomies of interest repeatedly.

the myriad ways in which these slight asymmetries can be expressed are undoubtedly not fully described and therefore not yet even known to investigators. In short, we either do not study it, do not experimentally control for it or do not know where to study it in our model organism of choice (Again, for an exception see *C. elegans*: Bauer Huang et al., 2007; Bertrand, Bisso, Poole, & Hobert, 2011; Chuang et al., 2007; Nakano, Ellis, & Horvitz, 2010; Nakano, Stillman, & Horvitz, 2011; Sulston & Horvitz, 1977; H. Suzuki et al., 2008)). As a result, the neuroscience community largely does not know the extent or nature of asymmetries in vertebrate central nervous systems.

Overcoming the knowledge gap: model organisms

Despite the bleak picture I have painted in terms of our relative ignorance about the organization of asymmetries in non-human vertebrate model systems, there are quite a few reported asymmetries across modalities and species. For example, visual stimuli have been documented to be lateralized in human (Aljuhanay, Milne, Burt, & Pascalis, 2010; Bruyer & Schweich, 1987; Proverbio, Brignone, Matarazzo, Del Zotto, & Zani, 2006; Rossion et al., 2003) and in non-primate vertebrates species (George, Hara, & Hessler, 2006; Peirce & Kendrick, 2002). Rhesus macaques (Hauser & Andersson, 1994), mice (Ehret, 1987) and songbirds (Phan & Vicario, 2010; Poirier, Boumans, Verhoye, Balthazart, & Van der Linden, 2009; Voss et al., 2007) have lateralized dominance for recognizing or processing species-specific vocalizations. However, while the list of behaviors or processes that are the dominion of one hemisphere or the other grow yearly,

and interest in this field is growing (Figure 1.2, 1.3), the vast majority of studies have only investigated adult animals. As a result, they have studied animals once the behaviors were *already* lateralized to one side of the brain or the other. *When* lateralized behaviors arise and, secondly, *how* they arise is almost completely unknown to us and is the central interest of my thesis. In vertebrates, there are two notable systems that may prove fruitful for the particular understanding of when and how behaviors become the dominion of one hemisphere or another: visual behavior asymmetries in chickens and pigeons, and song production in songbirds.

The chicken and pigeon as models for the ontogeny of brain asymmetry

Visual behavior in the domestic chick (*Gallus gallus*) and pigeon (*Columbia livia*) have been powerfully studied as models to understand the ontogeny of lateralization. The experiments performed utilize three advantages in these model organisms. First, the embryos can be easily studied and manipulated because they are encased within large eggs that can be kept in laboratory incubators. Secondly, 99% of efferents from the eye of birds project to the contralateral hemisphere (by contrast only 55% of human eye efferents project to the contralateral hemisphere) and birds do not have a corpus callosum, thereby rendering visual information entering one eye the near sole computational entity of one hemisphere, making birds almost natural split-brain patients. Thirdly, in both species, there is a clear asymmetry in experience by both sides of the brain whereby later staged embryos characteristically turn to the

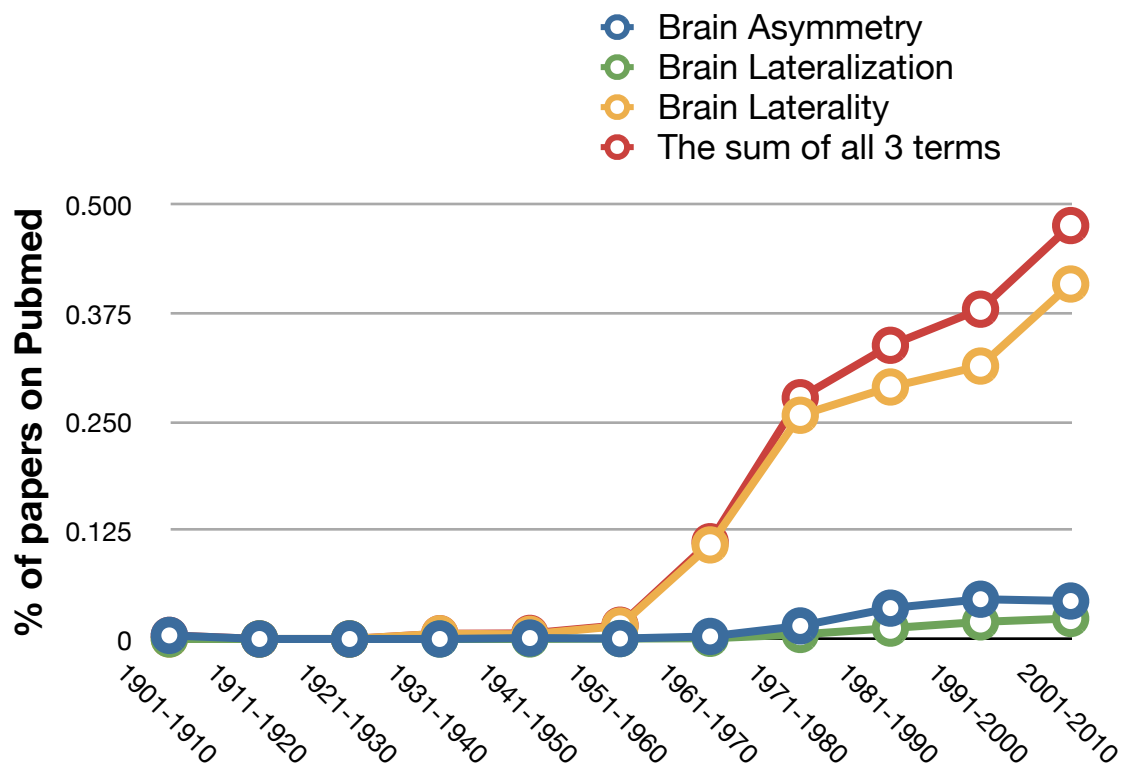


Figure 1.2: The percent of all life-science papers published on brain asymmetry is increasing. A Pubmed search for the terms ‘brain asymmetry’, ‘brain lateralization’, and ‘brain laterality’ in ten year intervals revealed steady growth in the percent of papers published containing these terms in the title or abstract. Notably, the increases in publication percentage began in the 1960s, coinciding with Roger Sperry’s work on commissurotomy.

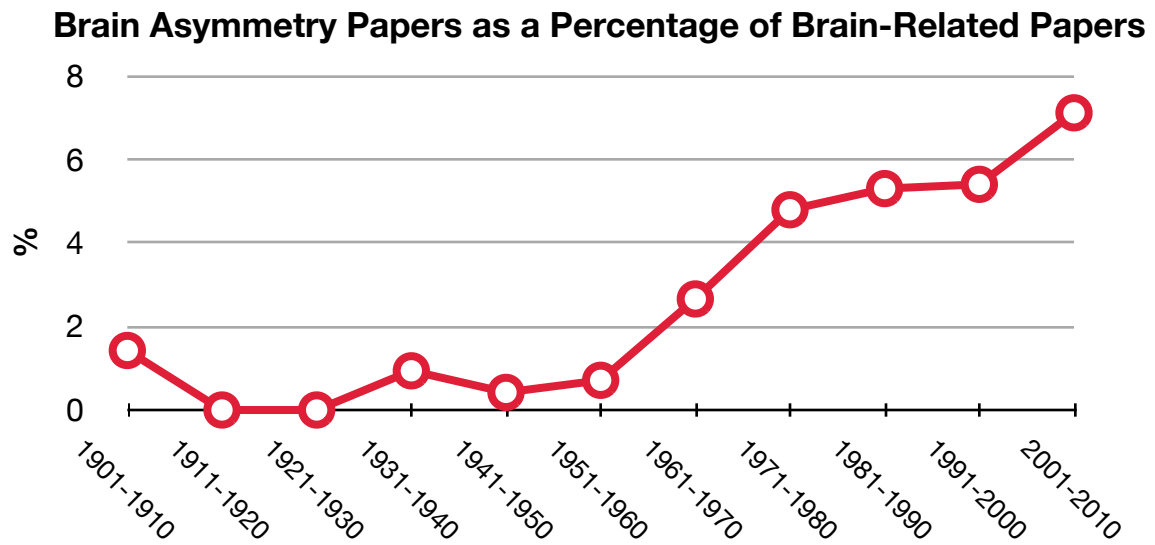


Figure 1.3: The percent of brain-related papers investigating or mentioning brain asymmetry is increasing. The percentage of all papers found on Pubmed by the search term 'brain' that were also found by the search terms 'brain asymmetry', 'brain laterality' or 'brain lateralization'. The percent of all brain-related papers that were about or made reference to brain asymmetry has increased every decade since 1960, suggesting a growing interest in or awareness of asymmetry in the brain.

right in the egg, with the left eye occluded first by the egg yolk and later its body, while the right eye is exposed to the light entering through the porous egg shell and sac membranes. Thus, each eye has differential exposure to light throughout development. The result is that occluding one eye leads to the silencing of the visual centers of the contralateral hemisphere. What a body of studies in these two species have shown is that asymmetrical exposure to light during a critical time in development leads to the establishment of visual object discrimination asymmetry. Chicks normally exposed to light while in the egg find food grains scattered in a background of small rock pebbles more readily using their right-eye/left-hemisphere than using the left-eye/right-hemisphere (Gunturkun, 1997; Rogers & Sink, 1988; Skiba, Diekamp, & Gunturkun, 2002). Intriguingly, eggs raised in the dark hatch chicks with no visual behavior asymmetry, and chicks raised in the dark whose left eyes are experimentally exposed to light, develop reversed visual behavior asymmetries (Rogers, 1990; Rogers & Bolden, 1991; Skiba et al., 2002). Thus, asymmetric light experience causes the lateralization of visual behavior in chicks and pigeons.

These authors, led primarily by Lesley Rogers at the University of New England in Magdwick Australia, have further shown that these behavioral asymmetries are accompanied by central visual system asymmetries, whereby more visual efferents from the left side of the thalamus project to the right Wulst region (Rogers & Deng, 1999; Rogers & Sink, 1988), a thalamofugal system that is equivalent to the geniculocortical system in mammals. Following the behavior

work, these visual system asymmetries are also established by light experience while in the egg and can be reversed or eliminated via identical light manipulations (Gunturkun, 1997; Rogers, 1990; Rogers & Bolden, 1991; Rogers & Deng, 1999; Rogers & Sink, 1988). In this way, the experimentally induced changes in normally asymmetric behavior co-occur with similar changes in neuroanatomy.

While the chick and pigeon models are the best currently understood vertebrate system for the ontogeny of lateralization, there are still a number of outstanding hurdles for scientists wishing to use these organisms for these experimental purposes. First, behaviors at the group level are indeed asymmetric, but none of the studied behaviors are completely or even overwhelmingly lateralized. In fact, most of the described behaviors really show a preference (typically ~60% to ~40%) for using one eye over the other, and some individuals show no or even reversed preferences, complicating studies aiming to understand the neural roots of these behaviors. Secondly, not all lateralized visual behaviors in the chicken are affected by light manipulation. For example, though dark-incubation abolishes lateralized food/background discrimination, these same chicks still asymmetrically discriminate familiar versus unfamiliar objects (Andrew, Johnston, Robins, & Rogers, 2004). Thirdly, the anatomical thalamofugal asymmetries in chicks are only present for the first 3 weeks of life, but adults nevertheless show behavioral asymmetries similar to chicks, thus complicating, if not altogether weakening, the form-function link proposed by

these authors. Finally, how and where within the visual system these asymmetric behaviors are processed is completely unknown. While there are outstanding problems still to be resolved, the chick and pigeon studies have greatly increased our understanding of how asymmetries in the brain may arise through environmental influences during critical periods (See Figure 1.1 for a review on the emergence and maintenance of asymmetries in the brain and periphery).

If the limitations in human studies on handedness do not allow us to understand *when* peripheral asymmetries arise, and human language does not allow us to understand *how* behavioral asymmetries appear because of the inability to manipulate subjects, the chicken and pigeon studies at least partially ameliorate these issues. However, these models are themselves limited in being currently unable to let us understand where these behavioral asymmetries begin centrally. Another order of birds, namely, songbirds, may allow us to better discover these origins.

Brain asymmetry in songbirds

Birds have long fascinated scientists and casual observers alike because of their songs, their spectacular beauty, and our jealousy over how they get around. If the Wright brothers helped to get us past our earthly insecurities, and a handful of geneticists are working to understand the brightly-colored plumage of birds, the last 40 years have seen an explosion of engineers, ethologists, neuroscientists, developmental biologists, endocrinologists, geneticists,

physicists, and mathematicians working to unravel how a tiny 1 gram dinosaur brain is able to learn to reproduce vocalizations in a manner that is only found in a handful of species, most notably, humans.

Sound production

Oscine songbirds (suborder Passeri or Oscine in the Passeriformes) vocalize using a bipartate structure located at the tracheosyringeal junction called the syrinx (Figure 1.4B) which consists of 6 bilaterally paired muscles and modified cartilages. During vocalization, this structure controls the movement and tension of the medial and lateral labia (Figure 1.4A). Endoscopic observations in songbirds show that these labia are drawn into the airstream, form a slit, and vibrate during phonation, suggesting that these are the primary sound sources in songbirds (Goller & Larsen, 1997). Indeed, later experiments (Larsen & Goller, 1999) measuring the motion of the labia during sound production found that the dominant frequency of labial vibration and vocalization matched. Therefore, while other minor sound sources may exist (Goller & Larsen, 1997; Nowicki, 1987), the medial and lateral labia are widely thought to account for the majority of sounds produced during vocalization. Electromyography (EMG) of syringeal muscles during singing has been used in a number of studies to better understand the role of each syringeal muscle in phonation. The syringealis dorsalis and tracheobronchialis dorsalis (The dorsal muscles in Figure 1.4B) are responsible for drawing the labia into a closed position, preventing phonation, as electromyography (EMG) activity in only these muscles is correlated with full

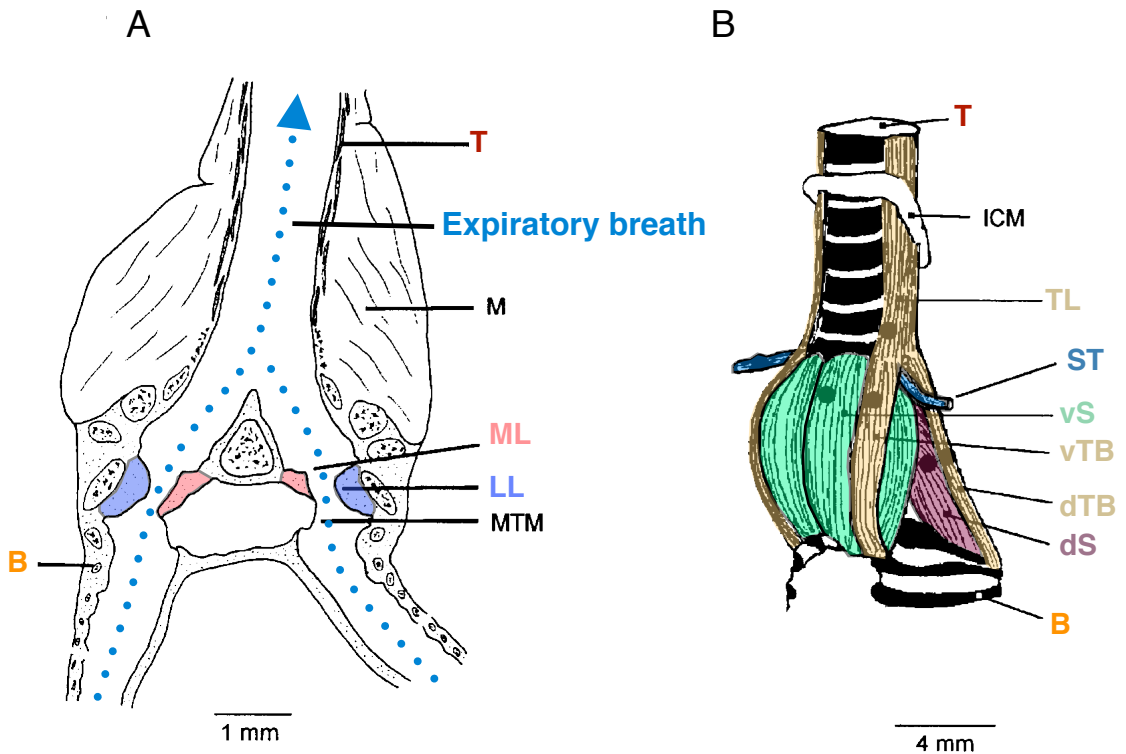


Figure 1.4: The syrinx. **A)** Frontal section of the syrinx. During phonation the medial (**ML**) and lateral labia (**LL**) are moved into the airstream (blue dotted line) forming a slit where they are set to vibrate. **B)** External view of the syrinx showing the dorsal and ventral syringeal muscles.

Abbreviations: **T** = trachea, **M** = Syringeal muscle, **ML** = Medial labium, **LL** = lateral labium, **MTM** = Medial tympaniform membrane, **B** = bronchus, **ICM** = membrane of the interclavicular air sac, **TL** = m. tracheolateralis, **ST** = m. sternotrachealis, **vS** = m. syringealis ventralis, **vTB** = m. tracheobronchialis ventralis, **dTB** = m. tracheobronchialis dorsalis, **dS** = syringealis dorsalis. Figure from (Suthers et al., 1999), with modifications.

ipsilateral closing (Goller & Suthers, 1995; Goller & Suthers, 1996b; Larsen & Goller, 2002). However, the role of these dorsal muscles is not just to turn phonation on or off, but also to set the labia into a phonatory configuration, as direct electrical stimulation of the dorsal syringeal muscles in anesthetized brown thrashers and cardinals leads to the adduction of the medial and lateral labia (Larsen & Goller, 2002). Thus, the dorsal muscles can be thought of as the muscles in charge of getting the musical instrument ready to be played or not. However, these muscles only play a minor role in the spectral qualities of song. The syringealis ventralis (The large ventral muscle in Figure 1.4B) on the other hand, has a large influence on the spectral properties of song, presumably accomplished by varying the tension of the labia (Suthers & Zollinger, 2008). Or, in other words, it plays the instrument. These large muscles are the only muscles whose EMG activity is highly correlated with frequency modulation (FM); as FM increases, EMG amplitude increases and when FM decreases, EMG amplitude decreases (Goller & Suthers, 1996a). Also, the EMG amplitude increases exponentially as fundamental frequency of song increases (Goller & Suthers, 1995; Goller & Suthers, 1996a). While these are not all of the characteristics of any call or song, it is clear that each muscle in the syrinx plays a distinct role in the spectral qualities of any sound produced.

Critically for the denervation experiments presented in this thesis, each half of the syrinx is innervated by the ipsilateral tracheosyringeal nerve (Figure 1.5; Nottebohm, Stokes, & Leonard, 1976). The tracheosyringeal branch of the

hypoglossal nerve is made of efferents emanating from only the ipsilateral tracheosyringial portion of the hypoglossal motor nucleus (nXiits or n12ts) in the hindbrain (Nottebohm et al., 1976). This nucleus in turn is innervated by the ipsilateral midbrain and forebrain motor nuclei. Thus, the songbird syrinx is functionally two vocal organs, each innervated predominantly by the ipsilateral hemisphere.

Vocalizations in songbirds require more than the coordinated activity of the muscles of the syrinx: Breath is necessary to set the syringeal labia into vibration. Sound production is first initiated by the generation of a high positive air sac pressure within the bird respiratory system. Unlike humans, the songbird respiratory system is composed of a number of bilaterally paired air sacs as well as a medial interclavicular air sac. Both inspiration and expiration are active processes, and vocalizations predominantly occur during expiration (Goller & Suthers, 1995; Hartley, 1990; Hartley & Suthers, 1990; Suthers, Vallet, Tanvez, & Kreutzer, 2004), which occurs with the contraction of intercostal and abdominal muscles (Hartley, 1990; Kadono, Okada, & Ono, 1963; Wild, 1993a). Also unlike humans, where the lungs are respiratorially separated, the air sacs on the right and left are connected through the interclavicular air sac and possibly other connections (McLelland, 1989). Thus, the respiratory system is not functionally lateralized, as contraction of one side of the expiratory muscles would cause air to flow to both sides of the syrinx (Nottebohm, 1971; Nottebohm & Nottebohm, 1976). Indeed, direct EMG measurements of expiratory muscles on both the left

and right side during lateralized vocalizations in the brown thrasher (see Appendix 4) showed no lateralization whatsoever at the level of expiratory muscles (Goller & Suthers, 1999). As the authors concluded, “lateralized song production is achieved through concurrent...unilateral activation of syringeal muscles in combination with bilaterally similar activation of expiratory abdominal muscles” (Goller & Suthers, 1999). This result is perhaps not surprising in light of the fact that respiratory premotor neurons in the right and left nucleus retroambigualis (RAm) project bilaterally to motor neurons that innervate the abdominal expiratory muscles (Wild, 1993a, 2004) and also receive bilateral inputs from key midbrain and forebrain premotor nuclei (Roberts, Klein, Kubke, Wild, & Mooney, 2008; Wild, 2004; Wild, Kubke, & Mooney, 2009).

Song development

The adult song that male birds produce to court females and defend territory is gradually perfected over months of trial and error learning and is learned by imitating a tutor. Song learning is thought to progress through three phases, each blending into the next (Figure 1.5). Song begins as a loose, rambling and unstructured set of vocalizations and ends as a highly structured and stereotyped song that closely resembles that of a tutor's. In a classic study on song development in chaffinches (see Appendix 4), William Homan Thorpe, who pioneered the use of sound spectrography in the study of birdsong, noted that early on birds produce soft meandering vocalizations that are akin to babies babbling, a stage of song now widely termed subsong (W. Thorpe, 1955; W. H. Thorpe & Pilcher, 1958). These vocalizations are then slowly organized and

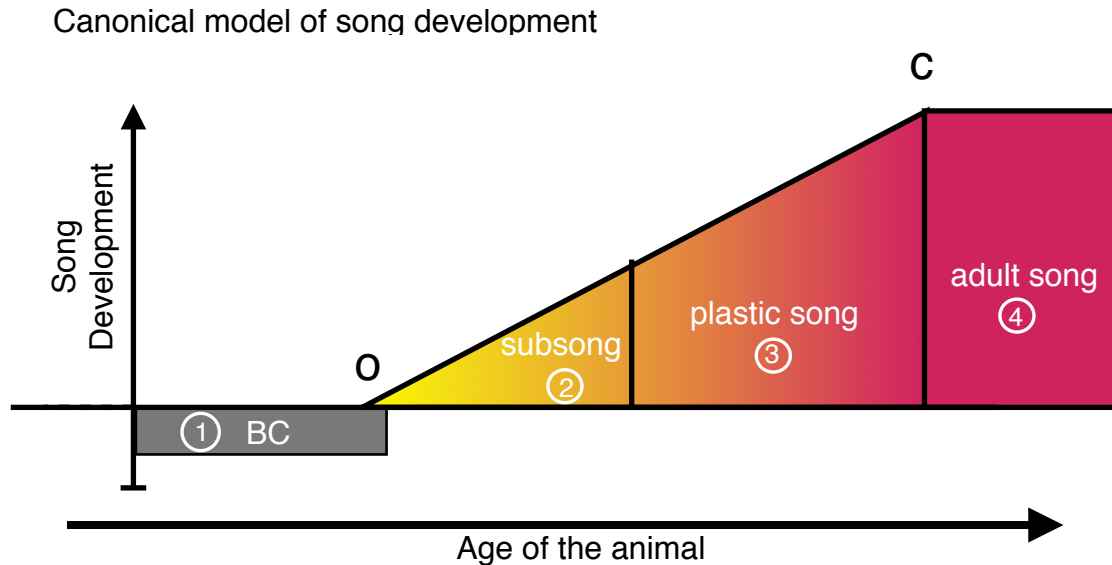


Figure 1.5: Vocal ontogeny and the currently accepted stages of song development. Food begging calls (**BC**; **1**) are overwhelmingly produced before any of the stages of song, though in some species they can co-occur with the earliest subsong (**2**). Begging calls are unrelated to song development in the canonical view of song development. The onset (**O**) of song development begins with subsong (**2**), is then followed by plastic song (**3**) and finally, when 'crystalized' (**C**), adult song (**4**). Adult song is thought to be modified throughout adulthood, though the changes are very slight and are thus not represented here. A gradient of color is used here to denote increased stereotypy and structure in song as development progresses. The gradient is additionally used to point out that each stage of song development smoothly transitions into the other and should thus be understood as a continuum that is divided into individual phases by identifiable feature changes for the convenience of study.

eventually more closely resemble an adult song, but still display variability between bouts and across days: a stage known as plastic song. When plastic song becomes highly stereotyped, it is said to “crystallize” into adult song, where it will remain largely unchanged. This same song may be sung for the remainder of the breeding season or, in fact, the animal’s life, depending on the life history of the songbird species. Thus, song is currently widely accepted to progress through three phases: subsong, plastic song, and adult song (Figure 1.5).

The song system

The songbird song system is composed of a subset of brain nuclei that collectively partake in the learning and production of song³. While we do not yet have a comprehensive understanding of how the system acquires and produces song, we know a fair amount about which players are involved, how they are connected to each other and what their roles are in learning or producing song (Nottebohm, 2005; Nottebohm, Kelley, & Paton, 1982; reviewed in: Nottebohm & Liu, 2010b; Nottebohm et al., 1976; Vicario & Nottebohm, 1988; Wild, 2004).

The song system can be simplified into two major pathways: 1) the anterior forebrain pathway, which is necessary for the learning but not production of song and, 2) the descending motor pathway, which is required for the production of learned vocalizations (Figure 1.7). Both of these pathways begin at

³ A feature of the song system that makes it so powerful to study and is often not mentioned is that for many of the nuclei in the system, there is no known function outside of the production, learning, or perception of song. The mammalian hippocampus does a lot more than memory and a lot more than place understanding, but song nuclei as far as we know just do song.

HVC (High vocal center of the nidopallium) and converge on the robust nucleus of the arcopallium (RA), thus making it the major premotor output of the forebrain. Nucleus RA subsequently projects to motor neurons in nXIIts (the tracheosyringeal portion of the nucleus of the twelfth cranial nerve; or n12ts) which project to the ipsilateral syrinx (Figure 1.7; Wild, 2004). Importantly, these two pathways exist within each of the two hemispheres but share no direct contralateral projections from any of the forebrain nuclei mentioned. While there are some contralateral projections at the level of the midbrain in nuclei not pictured (M. F. Schmidt, 2003a; M. F. Schmidt, Ashmore, & Vu, 2004; Wild, 2004), the two pathways shown above lie relatively isolated from one another in their own hemisphere, later projecting to ipsilateral motor neurons in the hindbrain which control only their ipsilateral syrinx (Nottebohm et al., 1976). Thus, incredibly, each syringeal half is predominantly controlled by song nuclei on the ipsilateral side (Nottebohm et al., 1976; Wild, 1997). However, songs that utilize both sides of the syrinx are not the result of two independent instruments playing with little regard for the other, but rather the highly orchestrated motor activity of both sides of the brain (Ashmore, Bourjaily, & Schmidt, 2008; M. F. Schmidt, 2003b; M. F. Schmidt et al., 2004; Vu, Schmidt, & Mazurek, 1998; Wang, Herbst, Keller, & Hahnloser, 2008). Thus, the two sides of the songbird brain, while largely controlling ipsilateral motor output, are kept coordinated by a series of midbrain nuclei.

The anterior forebrain pathway (AFP) is necessary for the learning of song predominantly through the introduction of variability into the vocalizations. The role of the AFP in learning is clear, as lesions to LMAN (lateral magnocellular nucleus of the anterior nidopallium) during song learning lead to premature crystallization consisting of a few simple syllables (Bottjer, Miesner, & Arnold, 1984; Olveczky, Andalman, & Fee, 2005; Scharff & Nottebohm, 1991) while LMAN lesions in adults cause little change to the gross structure of song (Bottjer, Halsema, Brown, & Miesner, 1989; Bottjer et al., 1984; Nordeen & Nordeen, 1993; Scharff & Nottebohm, 1991). Furthermore, variability in LMAN neuron activity correlates with syllable structure variability (Hessler & Doupe, 1999; Kao, Doupe, & Brainard, 2005) and introduction of variable activity into LMAN of singing birds drives increased song variability (Kao et al., 2005). These studies collectively implicate LMAN as a source of variability in the song of young birds, where this purposeful injection of randomness into the song system is thought to aid in the “motor exploration” important for learning how to reproduce a tutor’s song (Brainard & Doupe, 2000, 2001; Hessler & Doupe, 1999; Kao & Brainard, 2006; Kao et al., 2005; Morrison & Nottebohm, 1993). As the animal develops and its song more closely approximates that of its tutor, these inputs are reduced, allowing for stereotyped vocal productions. Thus, the progression of song learning (Figure 1.6) can be thought of as the transfer of functional dominance from the anterior forebrain pathway to the descending vocal motor pathway.

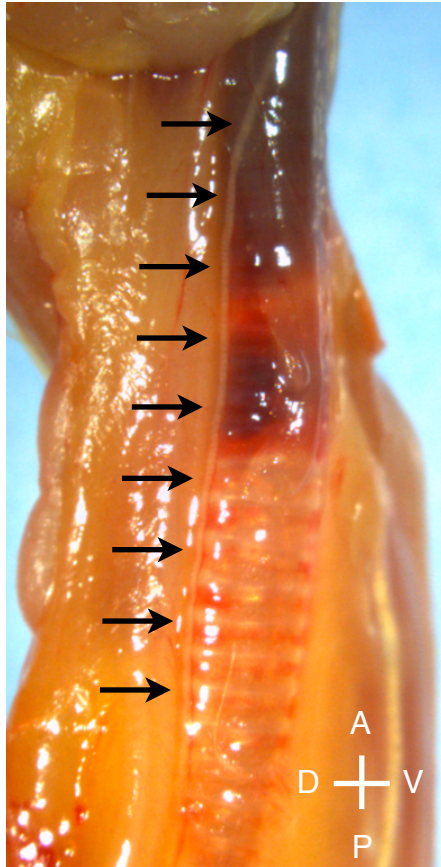


Figure 1.6: The tracheosyringeal nerve. The right tracheosyringeal nerve is visible running along the trachea (the white tissue highlighted by arrows). The left nerve is found on the opposite side of the trachea. A = anterior, P = posterior, D = dorsal, V = ventral.

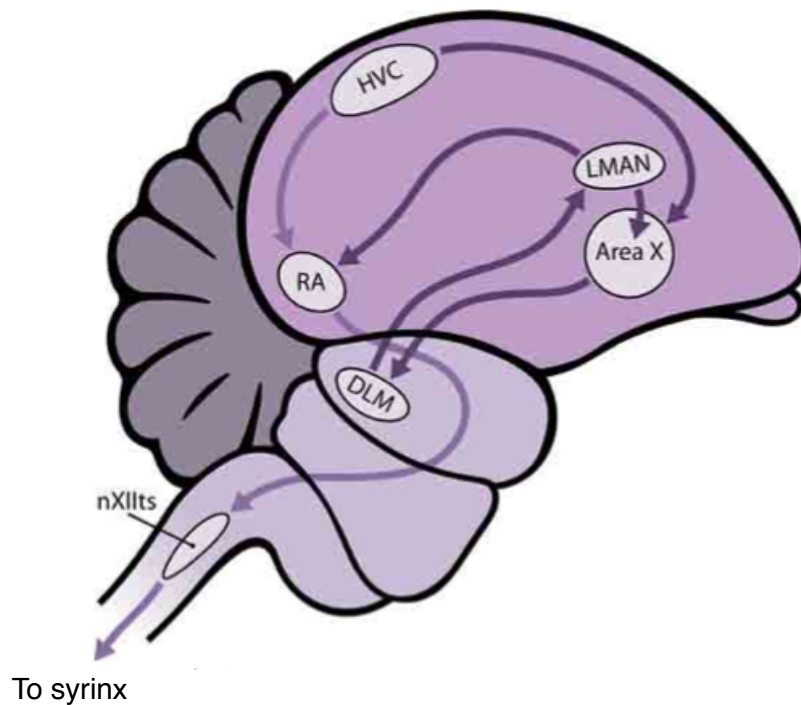


Figure 1.7: The oscine song system. This diagram depicts the major brain areas used in the production and learning of adult song. While a single hemisphere is shown above, these nuclei are found bilaterally, and there are no contralateral projections from any of the midbrain or forebrain nuclei shown above. Two pathways are depicted (light versus dark purple arrows), both emanating from HVC and ultimately converging on nucleus RA, the major premotor output of the forebrain. The anterior forebrain pathway (dark purple arrows; HVC→Area X→DLM→LMAN→RA) is necessary for vocal learning but not for the production of crystalized song. The descending motor pathway (light purple arrows; HVC→RA→nXIIts) is essential for the production of song. Motor neurons in nucleus nXIIts in the brainstem innervate the ipsilateral syrinx. Figure originally from (Nottebohm, 2005) with minor modification.

The descending vocal motor pathway (HVC→RA→nXIIIts) is critical for the production of learned vocalizations and is thought to control the timing of song. Individual HVC→RA projection neurons fire in a single 6ms window within the song (Hahnloser, Kozhevnikov, & Fee, 2002) and these neurons are thought to be connected in a synaptic chain (Long, Jin, & Fee, 2010), resulting in neurons or groups of neurons bursting sequentially through the song motif. This organized firing pattern is thought to not only underly the production of a precise, stereotyped song, but also its timing and structure. In an especially elegant science experiment, Michael Long, at the time in Michael Fee's lab at MIT, inserted a peltier device into HVC to cool the area, in theory slowing synaptic signaling, and as a result caused a stretching of the song (Long & Fee, 2008). In other words, cooling HVC led to an animal singing s-l-o-w-e-r! Warming HVC led to a faster song rendition, together suggesting that one of the roles of HVC in song is to keep the beat. However, HVC does more than act as a metronome, it also maintains the structure of the song. Bilateral lesions of HVC in zebra finches cause their songs to lose structure and stereotypy (Aronov, Andalman, & Fee, 2008). RA neurons, for their part, burst heavily throughout song and at multiple places within individual syllables (Leonardo & Fee, 2005; Yu & Margoliash, 1996). This pattern of firing is highly stereotyped, aligning with acoustic features of song with a precision of ~1ms (Chi & Margoliash, 2001; Yu & Margoliash, 1996). It is this precise firing pattern in RA that HVC drives with similar precision and LMAN makes more variable by its semi-random inputs to RA.

The features of song development have been described in considerable detail in zebra finches and canaries (Deregnaucourt et al., 2004; Guttinger, 1985; Nottebohm, 1991; Nottebohm et al., 1990; Nottebohm & Liu, 2010a; Nottebohm, Nottebohm, & Crane, 1986; Nottebohm, Nottebohm, Crane, & Wingfield, 1987; Tchernichovski, Mitra, Lints, & Nottebohm, 2001; Wilbrecht & Nottebohm, 2003). In zebra finches, increases in stereotypy and song structure nicely parallel the anatomical development of the brain. Projections into RA from LMAN are present as early as post-hatch day (P) 15 while HVC does not project into RA until P30-35 (Mooney & Rao, 1994). Even then, HVC→RA inputs are weak throughout subsong and do not significantly contribute to song production (Aronov et al., 2008; Aronov, Veit, Goldberg, & Fee, 2011). Thus, the strengthening of HVC→RA inputs and concurrent weakening of LMAN→RA inputs occur slowly, with minimal HVC→RA input early in song development.⁴

The canary

The domestic canaries (*Serinus canaria domestica*) that are pets in homes and model organisms for vocal learning and neurogenesis in laboratories throughout the world, were first bred in captivity in the 17th century. Wild Atlantic

⁴ An important note: While there is great similarity in the song system of canaries and zebra finches, there may be developmental differences in the timing of events described above. To my knowledge, no comparable work has been carried out in canaries. Nevertheless, while the dates may be different, the change in RA input emphasis from LMAN to HVC is likely similar.

canaries (*Serinus canaria*) are endemic to the Canary Islands⁵, Azores and Madeira, and were originally brought to mainland Europe by Spanish sailors. There, selective breeding practices led to a wide variety of strains selected for physical attributes and/or song qualities. While wild canaries are greenish in color (Figure 1.8A), there are now domestic strains that are yellow, orange, white, black, and red (For examples see figure 1.8B - F). Some have been bred for an assortment of plumage morphology variations (Figure 1.8B, C). Still others like the waterslager canary were selected for the subjective beauty of their song (Figure 1.8F). The waterslager strain of song canary that I used in this thesis work evolved through selection by breeders in the small town of Mechelen in Belgium as early as 1713. The name waterslager comes from the characteristically bubbling-water-like sounds in their song⁶. Waterslager canaries have selective hearing difficulty at high frequencies which may be responsible, in part, for the frequency band of their song, which has lower fundamental frequencies than those found in other strains (Gleich, Dooling, & Manley, 1994; Gleich, Klump, & Dooling, 1995; Okanoya & Dooling, 1985, 1987; Okanoya, Dooling, & Downing, 1990). This is the strain of canary brought over from Belgium in the late 1960s to establish the Rockefeller Field Research Center's canary colony in Millbrook, New York and which all the experiments described in

⁵ Interestingly, canaries are named after the Canary Islands, not the other way around. In fact, the island's name is derived from the Latin name *canariae insulae*, meaning "island of dogs."

⁶ The origins of the name in fact come from the Flemish term *waterslag*, literally meaning 'water beat'!

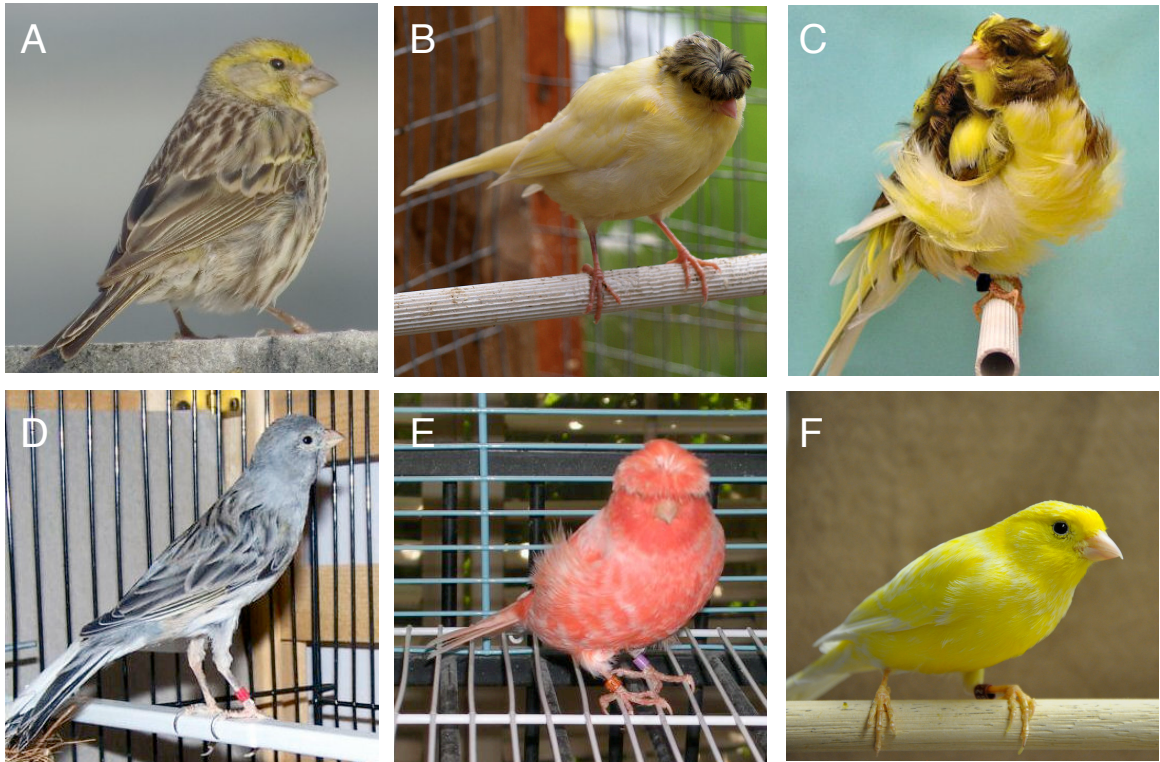


Figure 1.8: Wild and domestic canary strains. **A)** Atlantic canary. This is the wild species that was domesticated and selectively bred to give rise to the other strains. **B)** Gloster Fancy canary. **C)** Parisian Frilled canary. **D)** Spanish Timbrado canary **E)** Stafford canary. **F)** Waterslager canary. This is the strain used in the work presented in this thesis.

this thesis utilize. No new birds were added to the original stock of some 12 pairs, so the birds have been close bred for the last 40 plus years.

The canary's song

The adult waterslager canary song (Figure 1.9) is composed of syllables repeated numerous times to form a "phrase." The singer then changes syllable type and performs a new phrase and so on until the termination of song. The number of syllable repetitions within a phrase vary as do the number and order of phrases. The simultaneous production of two-voice sounds, where each side of the syrinx simultaneously produces a sound (say, the left syrinx performing an ascending whistle and the right syrinx an descending one) is rare in domestic and waterslager canaries but common in other species (Allan & Suthers, 1994; Suthers, 1990; Suthers, Goller, & Hartley, 1994). The fundamental frequency of the adult song in waterslager canaries is typically below 4 kHz.

Peripheral asymmetry in song production

Before we understood where birdsong was produced centrally, Fernando Nottebohm recognized that he could assess the contribution of each half of the syrinx to song by unilaterally sectioning the tracheosyringeal nerve. His studies of chaffinches (Nottebohm, 1971), canaries (Nottebohm & Nottebohm, 1976), and white crowned-sparrows (Nottebohm & Nottebohm, 1976; see Appendix 4) and replicated by others in white-throated sparrows (Lemon, 1973; see Appendix 4) and java sparrows (Seller, 1979; see Appendix 4) showed that left denervations

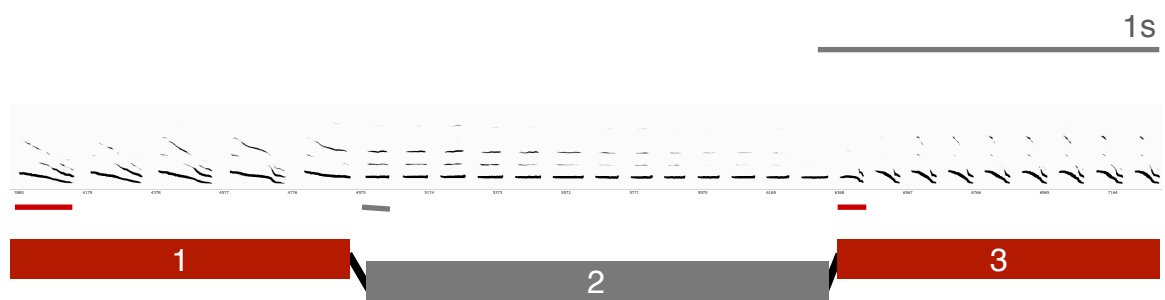


Figure 1.9: Snippet of adult canary song. Syllables (the first produced of each type is underlined) are repeated multiple times to form a phrase. Three phrases of a song shown above. Solid red and gray bars denote each phrase. Phrases are strung together to form a song. Note that the number of syllable renditions differs between each syllable type.

of this nerve had larger effects on song than right denervations. The effects when looking at the resulting sonograms were striking, appearing as though left denervations caused the replacement of song syllables with bursts of noise or silence (Figure 1.10). In fact, when Nottebohm, and others (Hartley & Suthers, 1990), counted pre and post denervation syllables, ~10% of adult song syllables were affected by right denervations while left denervations affected ~90% of song syllables. In another experiment, the effects of song following the unilateral sectioning of the tracheosyringeal nerve (Figure 1.6) were not further complicated by additionally sectioning the hypoglossal roots on the same side, thus indicating that the effects of unilateral nerve cuts are due primarily to the tracheosyringeal nerve that innervates the syrinx, the birds vocal organ.

Nottebohm went further and tested the potential for song recovery by the right hemisphere by left-denervating birds throughout early song development and then analyzing whether the right denervations had an effect on adult song. Left denervations before the onset of song development in 1-4 week old canaries resulted in right-hypoglossal control of song in those birds where the nerve did not regrow. The adult song of these early-denervated birds was of a similar quality (number of syllables, frequency characteristics) as intact 1 year old canaries. On the other hand, these same left-denervations during plastic song, when song development is well underway, resulted in much poorer quality songs under right hypoglossal control (Nottebohm, Manning, & Nottebohm, 1979).

Thus, there is substantial potential for reversal of hemispheric dominance, but with diminishing ability as song develops.

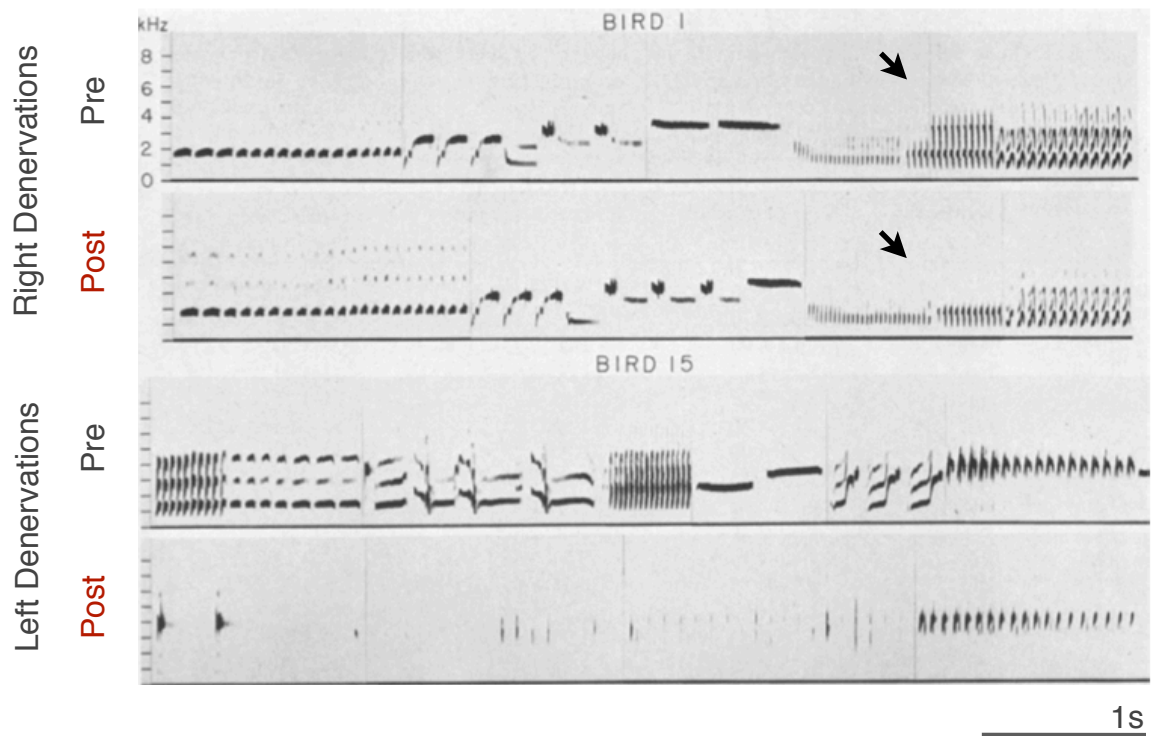


Figure 1.10: Left denervations of the tracheosyringeal nerve result in greater deficits than right denervations in adult canaries. Right denervations in adulthood resulted in the loss of some elements of syllables (arrows) but otherwise the song remained largely unaltered. Oppositely, left denervations caused a near complete loss of song with a few syllables remaining. Figure taken from (Nottebohm & Nottebohm, 1976) with slight modification.

The result of this body of experiments was the first described lateralized vocalization in non-humans, and the fact that, as for speech, it was a learned skill, made it of special interest. Moreover, data on song ontogeny and adult performance were relatively easy to collect and score, so this became a particularly attractive system in which to study how functional lateralization was expressed in the brain and emerged in the individual.

Cerebral lateralization in song

A few years after his original denervation work, Nottebohm described significant portions of the song system and undertook unilateral lesions in nuclei within it to assess the post-operative effects on adult song (Nottebohm et al., 1976). Again, the differences in the effects of left or right hemisphere lesions on song were striking. Left HVC lesions caused large disorganizations in the structure of song, with phrases all but disappearing and little to no syllable stereotypy (Figure 1.11; Nottebohm et al., 1976). Right lesions did cause syllable stereotypy to decrease slightly but otherwise the song remained largely unaffected (Riggs, Minuth, Nottebohm, Rossen, & Suki, 1975). This work was the first to present evidence of lateralization of vocal production in non-humans and highlighted the songbird as a model for the study of brain asymmetry and behavior. Since, many studies have described other ways in which lateralizations of behavior are expressed within the song system. For example, the right MLd (lateral mesencephalic nucleus), HVC and Area X all show significantly greater

BOLD responses for the bird's own song versus songs from other birds of the same species (conspecific song; Poirier et al., 2009). The results of this study suggest that auditory processing of song may be lateralized, with a right-hemispheric bias for the processing of a bird's own song, results of which have been replicated elsewhere (Voss et al., 2007). In a direct test of the lateralized response to song in the right hemisphere, multielectrode recordings in the caudal medial nidopallium (NCM), a cortical-like brain auditory area in songbirds, showed that auditory responses to conspecific songs were stronger on the right than the left NMC (Phan & Vicario, 2010). While more studies will need to be carried out to better define each hemisphere's role in song production or perception, the early results are suggestive of vast specializations within each hemisphere for a wide variety of song-related modalities including production and perception.

Recent reinterpretations of early results on peripheral asymmetries in canaries

In the past decade, there has been some rethinking of the interpretation of the results first gathered in waterslager canaries. Work in other songbirds, including brown thrashers (Suthers et al., 1994), grey catbirds (Suthers et al., 1994; see Appendix 4), brown-headed cowbirds (Allan & Suthers, 1994; see Appendix 4), and cardinals (Suthers & Goller, 1996; see Appendix 4) has shown that the left syrinx produced phonations below 3.6 kHz and the right syrinx contributing high frequency phonations above 2.5 kHz, with sounds of frequencies between 2.5-3.6 kHz produced by either side from animal to animal.

Put differently, in songbird species that sing across a broad frequency range, the percentage of syllables contributed by each side of the brain/syrinx is correlated with the amount of high or low frequency syllables, where animals that sing predominantly low frequency syllables will appear to have more left-lateralization of song function than those which sing low and high frequency syllables equally. The waterslager strain of canary used in all of Fernando's early work are selectively deaf to high frequencies, and the authors of a study (Suthers et al., 2004), led by Rod Suthers and Michel Kreutzer wondered whether the song would be as overwhelmingly lateralized in canaries if they used a non-hearing impaired strain. In this particularly informative study, they performed unilateral bronchial plugging (thereby disallowing one side to phonate as no air could escape through it) on an outbred strain of domestic canaries, which show no selective hearing loss and which sing across the songbird frequency range, including at much higher frequencies than the waterslager strain. What their work showed was that, indeed, canaries were no different than other songbirds. Specifically, the low and high frequency syllables were under left and right control, respectively (First suggested in: Nottebohm et al., 1979; Nottebohm & Nottebohm, 1976; Nottebohm et al., 1976), and that canary strains which sing across the frequency range do not show as much lateralization of song production as the waterslager.

These authors concluded at the time that what is truly lateralized in the canary brain is not song but rather frequency. Thus, the argument follows that the

deafness of our strain of canaries to high frequencies is the cause for their dramatic left-hemispheric dominance of song production. If the waterslagers could only hear high frequencies, they would produce high frequency syllables and those would be produced by the right hemisphere/right-syringeal half. However, since waterslagers cannot hear high frequencies, our birds sing almost exclusively with the left. This high/low frequency arrangement between the two sides of the brain confirming Nottebohm's early suggestion that the two sides may have their preferred frequency ranges after observing that high frequency elements or syllables were generally the only portions of song affected with right denervation and the only components that remained with left denervations (Nottebohm, 1971; Nottebohm et al., 1979; Nottebohm & Nottebohm, 1976; Nottebohm et al., 1976).

So, where does that leave us and lateralization of song in our canaries? There are a few key observations in deafening studies that may shed significant light and lead our thinking. Specifically, if the selective deafness to high frequencies in waterslager canaries pushes vocalizations to the left hemisphere because the left produces low frequency vocalizations which they can hear, this hypothesis would predict that completely deaf waterslager canaries or those without any auditory feedback experience in their lives should not have any lateralization of song. In one of Fernando's early papers on left hypoglossal dominance in canaries (Nottebohm & Nottebohm, 1976), he found the opposite: lateralization of song occurred even in birds that never had access to their own

auditory feedback and birds that had both cochleas removed early in life. These deafened birds never heard their own song and were still left-lateralized. Thus, while the lateralization of frequency production hypothesis likely explains why our birds sing predominantly with the left and not the right, this hypothesis does not explain or account for the deafening data. Moreover, unilateral lesion data in waterslager and domestic canaries clearly demonstrates that the two hemispheres are not acting symmetrically in the control of song, even in normal hearing birds. In both waterslager and, critically, domestic canaries, left, but not right, HVC lesions cause the loss of structure and stereotypy in adult song (Figures 1.11, 1.12; Halle, Gahr, & Kreutzer, 2003; Nottebohm et al., 1976). Additionally, in assessing the effects of unilateral HVC lesions on 12 song parameters in domestic canaries, left lesions significantly affected 8 parameters including song duration, repertoire size, the number of simple syllables, the number of complex syllables, and repetition rate. Right HVC lesions did not affect any of these characteristics (Michele Kreutzer, Fred Halle, in preparation, personal communication). Thus, while average frequency produced by each syringeal half appears to indeed be lateralized in songbirds whereby low frequencies are produced by the left and high frequencies by the right, these are layered on top of lateralizations of song structure control within the song system.

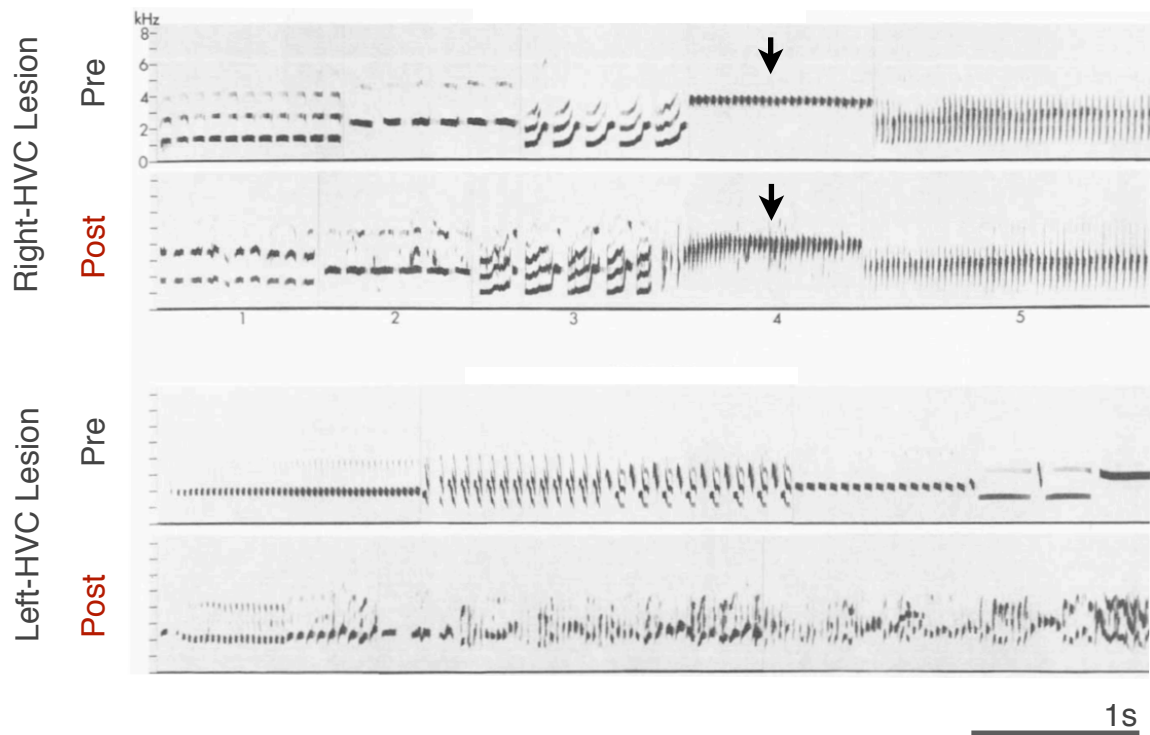


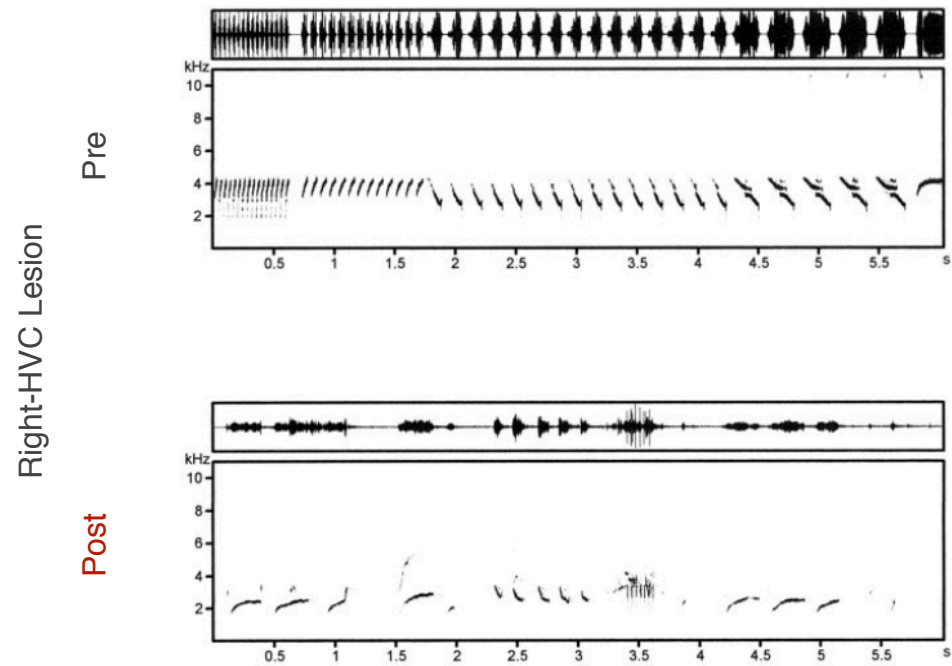
Figure 1.11: Lesions of the left HVC result in greater deficits than lesions to the right HVC in adult canaries. Unilateral lesions to HVC in adulthood resulted in some syllable loss, but the effects of left and right lesions on song are asymmetric. Right HVC lesions caused a slight decrease in the stereotypy in syllables, (arrows) but otherwise the song remained largely normal, with song structure remaining intact. Left HVC lesions caused a near complete loss of song phrase structure. Figure taken from (Nottebohm et al., 1976), with slight modification.

Figure 1.12: Lesions of the left, but not right, HVC result in structural loss of the song and syllable stereotypy in outbred domestic canaries.

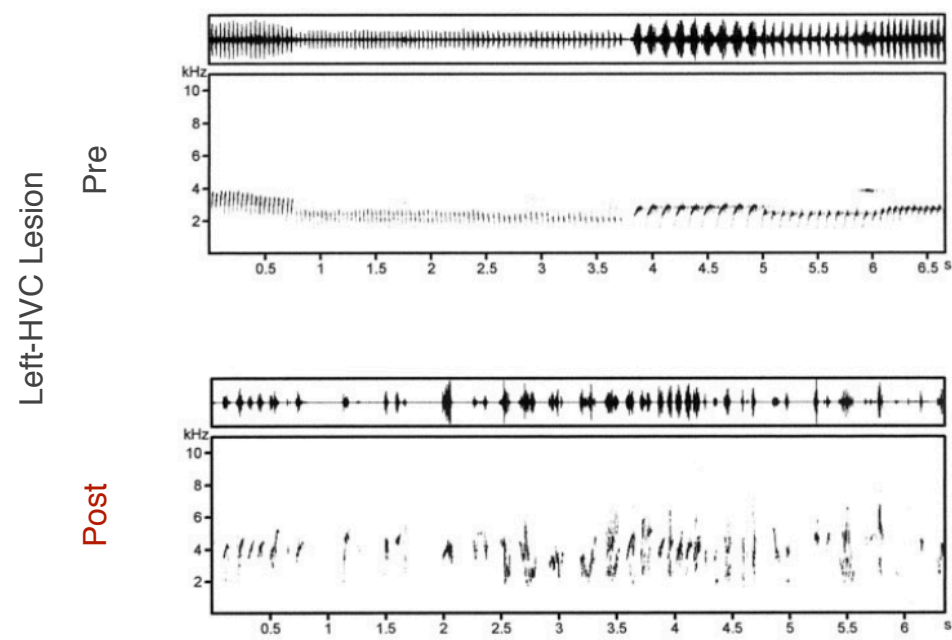
The effects of left and right lesions on song in an outbred strain of canary are asymmetric. **A)** Right HVC lesions caused significant changes in the adult song but phrase structure and syllable stereotypy are still largely present. Notably, it appears as though birds are no longer able to transition between phrases but instead produce them as disconnected islands of song. The number of syllable repetitions is also affected with right lesioned birds producing fewer syllables per phrase. **B)** Left HVC lesions in domestic canaries cause a near complete loss of song phrase structure and syllable stereotypy. The syllables of song following left lesions appear as almost random productions of sound, with some not at all resembling adult song syllables, perhaps akin to subsong. Right lesions result in phrases containing fewer syllables but with adult song-typical syllables still present. Phrase structure appears only mildly affected but transitions from one phrase to another now occur with significant lapses of silence. Figure taken from (Halle et al., 2003), with slight modification.

Figure 1.12

A



B



Goals of this thesis

I have tried to make it clear in the preceding review that while there is a large body of work on the nature of peripheral and neuroanatomical asymmetries and lateralized behaviors, some fundamental questions still remain unanswered. The question that I have tried to clarify how behaviors become the dominion of one hemisphere or another in vertebrates. Towards that goal, the aims of my thesis have been the following.

Goal 1. Establish a well-defined model for current and future studies by describing begging calls in canaries.

1. How does begging call ontogeny proceed?
2. What are the major call types produced during begging ontogeny?
3. How is each call used by individuals?
4. How are the major call types produced?

Goal 2. Identify when and how vocalizations become asymmetrically produced.

1. When do vocal asymmetries arise?
2. Do vocal asymmetries arise gradually or suddenly?
3. Can vocal dominance be reversed?
4. How does lateralization of vocal production becomes established?

Goal 3. Can the onset of the lateralization of begging calls be manipulated?

Chapter 2: Food begging calls in the canary

Nestling birds solicit food from parents by using begging displays that include vocalizations and exuberant movements such as wing flapping, upright postures and head movements. These vocal and visual displays act as signals of hunger and under normal conditions elicit parental feeding. For example, the hungrier a nestling is, the more it begs and the louder its begging, resulting in preferential feedings by parents (H. C. J. Godfray, 1991; R. M. Kilner & N. B. Davies, 1999; Kilner & Johnstone, 1997; Kilner, Noble, & Davies, 1999; M. L. Leonard & A. G. Horn, 2001; Lessells, Riebel, & Draganoiu, 2011; Price, Harvey, & Ydenberg, 1996; Smiseth & Moore, 2002; Soler, Soler, Martinez, & Moreno, 1999). Begging calls however, also include information that signals nestling identity (Buhrman-Deever, Hobson, & Hobson, 2008; Draganoiu, Nagle, Musseau, & Kreutzer, 2006; Levrero, Blanc, & Mathevon, 2012; Levrero, Durand, Vignal, Blanc, & Mathevon, 2009; McArthur, 1982; Reers & Jacot, 2011). Thus, feeding parents can use begging call signals to preferentially allocate scarce food resources. However, while this would suggest that increasing the production, amplitude, or rate of food begging calls would be evolutionarily advantageous for chicks, nests with more exuberant food begging are at increased predation risk from eavesdropping predators (Briskie, Martin, & Martin, 1999; Dearborn, 1999; D.G. Haskell, 1994; Leech & Leonard, 1997; Martin & Briskie, 2009). Thus, these two selection pressures must be balanced by both parents and offspring. For their part, feeding parents have evolved ways to modulate the begging

vocalizations of their offspring including begging solicitation calls and alarm calls. Begging solicitation calls are given by feeding parents in many species of birds as they arrive to the nest or while feeding nestlings and are effectively an 'on' signal (M.G. Anderson, D.H. Brunton, & M.E. Hauber, 2010b; J. R. Clemmons, 1995; Madden, Kilner, & Davies, 2005; Raihani & Ridley, 2007). Alarm calls on the other hand are given by parents from a distance at the perception of a threat to the nest and reduce or alter begging by nestlings, thereby acting somewhat as an 'off' signal (Anderson et al., 2010b; Davies, Madden, & Butchart, 2004; Platzen & Magrath, 2004, 2007). Therefore, young use begging calls to induce feeding by signaling nutritional need and identity and parents modulate the production of these calls and use them to preferentially allocate resources.

While there is clearly appreciated complexity in parent-offspring signaling in the literature (H. C. Godfray, 1995; H. C. Godfray & R. A. Johnstone, 2000; D. G. Haskell, 1999), the begging calls themselves have largely been thought of as relatively simple, unlearned, reflexive calls that are used by nestling birds only as a means to signal some trait or internal state and thus do not need to change across time. In other words, as begging calls are elicited during feeding and the mechanism of feeding -a parent placing food into an open beak- does not change across this period of life, developmental changes in the features of begging calls might be predicted as unnecessary. Moreover, the results of begging calls, namely being fed, are signal-dependent, meaning that begging calls from one species largely do not elicit feeding by parents from another unless the

heterospecific calls are copied (M.G. Anderson, D.H. Brunton, & M. E. Hauber, 2010a; M.E. Hauber, 2003; M. E. Hauber, 2003; R.M. Kilner & N.B. Davies, 1999; R. M. Kilner & N. B. Davies, 1999) and therefore these calls have been thought of as having great evolutionary constraints on them. In effect, if begging calls are very simple vocalizations produced by very immature birds and these calls do not *need* to change across development nor *should* they change, they are unlikely to. However, a number of reports have documented developmental changes in the structure of begging calls (Anderson et al., 2010a; J. Clemmons & Howitz, 1990; Liu, Wada, & Nottebohm, 2009), including sexual dimorphism in begging calls in, for example, barn swallows (Saino et al., 2003; Saino, Mary De Ayala, Boncoraglio, & Martinelli, 2008; see Appendix 4), cowbirds (M. E. Hauber & C.K. Ramsey, 2003; Appendix 4), and chipping sparrows (Liu et al., 2009; Appendix 4).

The developmental dynamics of begging calls and the underlying neuroanatomy have also been largely ignored because studies using songbirds predominantly focus on the development of vocal learning or the neural mechanisms of adult song and begging calls are widely held to be unrelated (Though see: F., 1972; Liu et al., 2009). In many songbird species, begging calls stop days to weeks before any subsong vocalizations are produced and therefore this temporal gap in vocalizations has led to the interpretation that food begging calls and subsong are different developmental vocalizations not having much to do with one another, akin to a baby crying for food and later language

development. Thus, almost all studies on food begging calls have focused on the ethology of food begging and/or the feeding parent's reaction to it, with less of a focus on the neurobiology underlying begging calls, with one notable exception. Wan-Chun Liu, an Assistant Professor in our laboratory, found that the begging call of chipping sparrows was clearly sexually dimorphic at P21 in a number of features including call duration, entropy, and pitch goodness (Liu et al., 2009). Moreover, he documented that deafening of male, but not female, P18 - P28 sparrows led to changes in the structure of begging calls. Lastly, lesions to nucleus RA in P22 - 24 males reduce variability in the begging calls. Thus, Liu collectively showed that almost—independent birds, near the end of food begging, display some features of song development in their food begging calls including sexual dimorphism, influence of auditory feedback, and utilization of forebrain nuclei. The study suggested that begging calls and song development might be part of the same stream of vocal ontogeny.

The ontogeny of begging calls, an introduction

Looking at one nestling canary across begging call ontogeny, it is clear that a number of changes occur in the structure of calls produced while food-begging (Figure 2.1, underlined in red). Food begging calls can be elicited in post-hatch day (P) 4 - P5 hatchlings but were too quiet to be recorded in our set-up before P6 -P7 and are not shown. Initially, the bird produces quiet whistles of largely sustained frequency (Figure 2.1, P9 - P12). Within a few days however,

the call changes to contain two elements, consisting of a low amplitude whistle of varying length followed without a break by an inverted-U-shaped element of higher frequency and amplitude (Figures 2.1, P13 - P17, 2.2, underlined in red). This call structure, which persists until the bird ceases to produce food-begging calls (typically P19 - P25), is widely seen in the majority of birds (Figure 2.2) and I shall refer to this vocalization as type A calls. Beginning around P16, a second distinct call type appears that is characterized by more marked and rapid frequency modulations (Figures 2.1, underlined in blue) that I shall refer to as B calls because they structurally appear to be different and also sound very different from the more typical A calls.

Experiment 1: Are food-begging calls similar across one day within nestling?

Overall, there appear to be pronounced changes in the structure of **type A** food-begging calls across even a single day during development. Such rapid changes might make it difficult to interpret the structural changes due to development as different from those induced by experimental manipulations such as denervation. Thus, I first sought to quantify how much variability was found across one day within individuals and also whether changes were gradual or abrupt. If calls are roughly stable across a 10-12 hour period, then recording one feeding session per day would suffice to capture that animal's calls at that age and would greatly simplify data collection. Moreover, later experimental manipulations such as denervations can then be analyzed comparing a single feeding session before and then after the manipulation. On the other hand, if these calls change dramatically across a day, then any comparisons before and after surgery, for example, must include multiple sessions so as to better encompass the variability of the animal.

A cursory look at the pitch of **type A** food-begging calls in two individuals across development visualized by feeding session revealed a trend over time of decreasing pitch but very large within-day variability (Figure 2.3). To quantify if this intra-day variability was statistically significant, multiple feeding sessions of

Figure 2.1: The ontogeny of begging calls of one individual. The food-elicited calls of one individual from P9 - P18 are displayed above. P9 - P18 denotes age of individual at time of recording. The structure of A calls, underlined in red, change over time (P9 - P16). Type B calls, underlined in blue, are structurally distinct calls characterized by rapid frequency undulations that first appear later in development (P16). Later B calls (P18) appear distinctly different from A calls and from earlier produced B calls (P16). All recordings are from bird # 204(2009). Note that the sonogram images at P9 and P16 were image processed (IP) to better visualize quiet vocalizations. Please refer to the 'Image Processing Section' of the thesis for specific information.

Figure 2.1

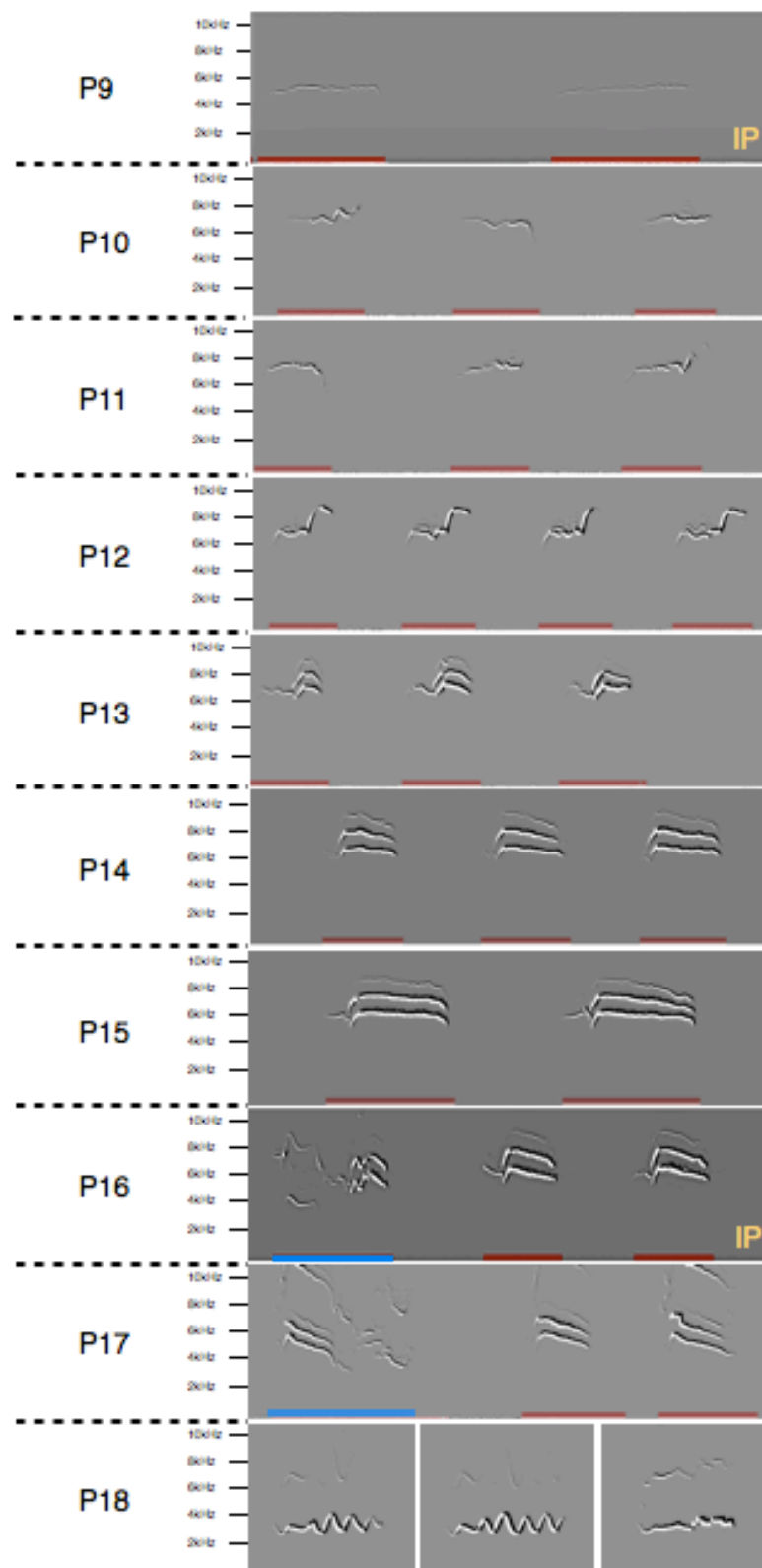


Figure 2.2: Food-begging calls differ between individuals but have similar components. The typical food-begging calls (underlined in red) of eight P14 canary nestlings are shown. The X-axis displays time in milliseconds (see text below sonograms) and all sonograms are scaled similarly. Note that though the A call can be highly dissimilar between animals, they all share a common pattern consisting of two elements; the call starts with the first element, a low amplitude whistle (white arrow) which is followed by a second higher amplitude element (light blue arrow) whose frequency rises, plateaus and often falls. The first element is sometimes short as in bird 122(2011) or sometimes long as in bird 25(2011). The second element has similar variability.

Figure 2.2

Bird Number:

Type A Food-Begging Calls:

10(2010)



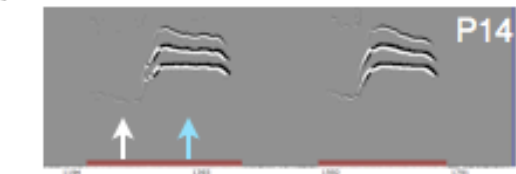
22(2011)



25(2011)



84(2011)



92(2011)



122(2011)



219(2011)



278(2010)

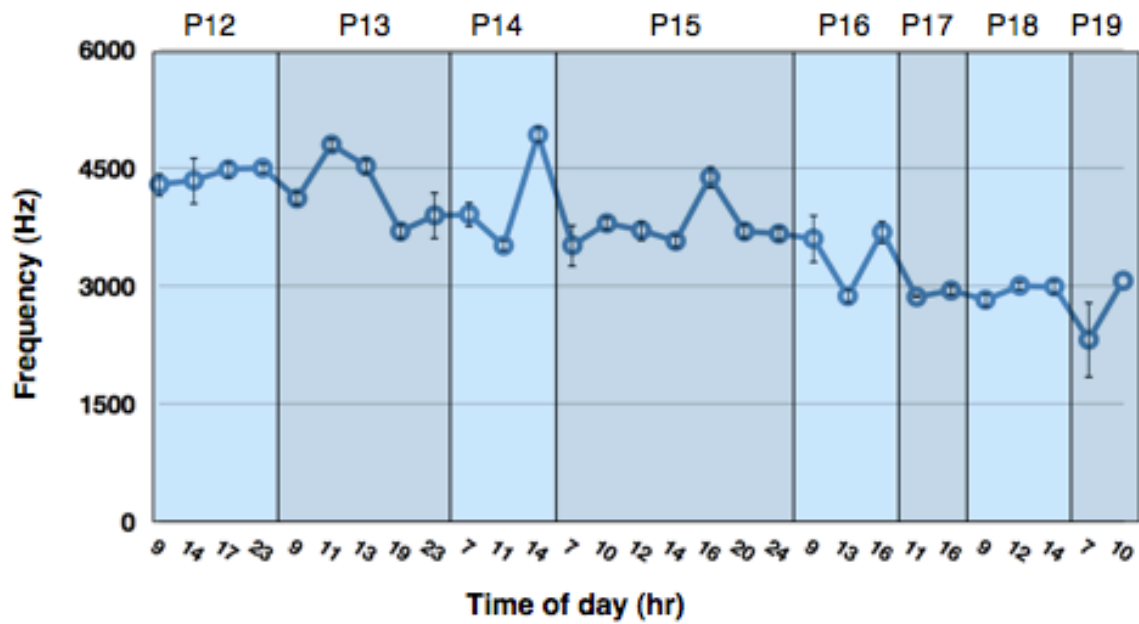


Figure 2.3: Average pitch in two individuals decreases across development but there is great variability within any single day. Average pitch at each feeding session across multiple days in two canary nestlings. Averages \pm Standard Error shown. Days are represented by alternating colored backgrounds to aid identification. Age of bird at day of recording is found above each day block. The time of feeding is shown across the X-axis (0-24 hours). Note that the time of recordings was different day to day and that some days had more or less recordings than other days. **A)** Bird 201(2010). **(B)** Bird 202(2010).

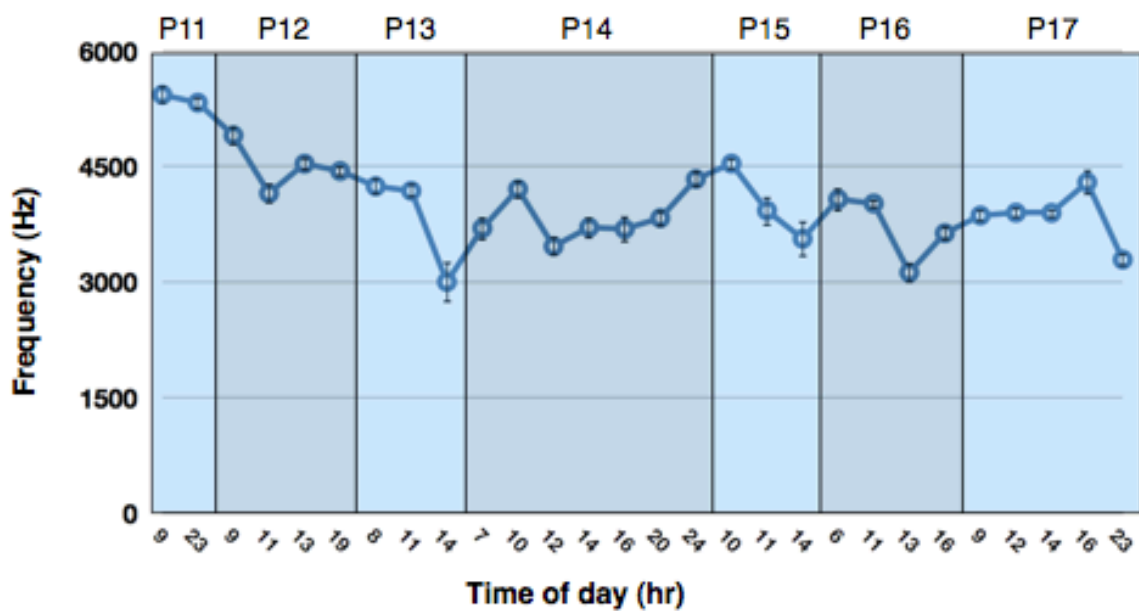
Figure 2.3

Average Pitch

A Bird 201(2010)



B Bird 202(2010)



four P16 canaries, 2 male and 2 female, were recorded throughout the day. Afterwards, every begging call within each feeding session was analyzed for call characteristics using Sound Analysis Pro (SAP). For methods on how begging was elicited, recorded, and calls analyzed, see 'General Methods for all Experiments' section. Feeding sessions were then compared to one another within each bird using a One-way ANOVA and Tukey's Multiple Comparison Test.

Results & conclusions:

A One-way ANOVA revealed that the average pitch of **type A** food-begging calls varied significantly within a day for every bird, **201F (bird #, sex):** $F(1, 5) = 10.79, p < 0.0001$, **219F:** $F(1, 8) = 5.920, p < 0.0001$, **208M:** $F(1, 5) = 6.167, p < 0.0001$, **120M:** $F(1, 9) = 8.836, p < 0.0001$. Tukey's Post-Hoc tests revealed significant differences between feeding sessions within each individual and are found in Figure 2.4. The same significant intra-day variability was found in every call feature analyzed (average call duration, average frequency, average frequency modulation, and average entropy) in every bird (data not shown). The results of this experiment show that there are robust intra-day changes in the characteristics of **type A** calls of canaries. Surprisingly, the changes appear to be directionally random from feeding session to feeding session. We might, for example, instead have expected a trait such as the average pitch of calls to decrease as the animal got older, simply due to the wider diameter of the developing vocal tract (bronchi, trachea), akin to the corollary frequencies of pan flutes and their diameter. As growth of a developing animal predominantly

proceeds in one direction if the animal is sufficiently fed (growth increases), pitch might have decreased across age smoothly. However, though the birds in this experiment were fully fed throughout the day, the changes in pitch did not follow the expected smooth pattern, suggesting that the calls were either not produced passively like a pan-flute, or that they are altered by internal states, environmental stimuli, or any other reason we might conceive of but have not tested. Either way, the result of these large intra-day changes in call features is that multiple feedings must be recorded preceding and following any surgery or manipulation to better assess effects.

Experiment 2: Are begging calls significantly different between individuals?

In experiment 1 I found that **type A** calls could differ significantly between feeding-sessions within an individual. Nevertheless, similar developmental trajectories as those pictured in Figure 2.1 are seen between birds in call morphology and I thus sought to quantify whether there was between-bird variability in call characteristics. To do so, I identified the first 10 **type A** food begging calls elicited in the first feeding session in five P16 males and analyzed each call for call characteristics using SAP. All birds were fed at the same time the night before to ensure roughly equal hunger levels. The values of each individual for each call characteristic were then compared to each other using a One-way ANOVA and Tukey's Multiple Comparison Test.

Figure 2.4: The average pitch of food-begging calls within an individual can change dramatically from feeding to feeding. The average pitch (Y-axis) of food begging calls recorded at each feeding session across one day in 4 individuals (A-D) are shown above. The time of each feeding is shown on the X-axis. Average begging call pitch changes significantly between feeding sessions in all birds. The birds represented are 201(2010)F **(A)**, 219(2010)F **(B)**, 208(2010)M **(C)**, 120(2010)M **(D)**. Note that not all feedings were recorded for every bird. A minimum of 35 calls are represented within a feeding session. Averages \pm Standard Error shown. * = $\leq p$ 0.05, ** = $\leq p$ 0.01, *** = $\leq p$ 0.001.

Figure 2.4

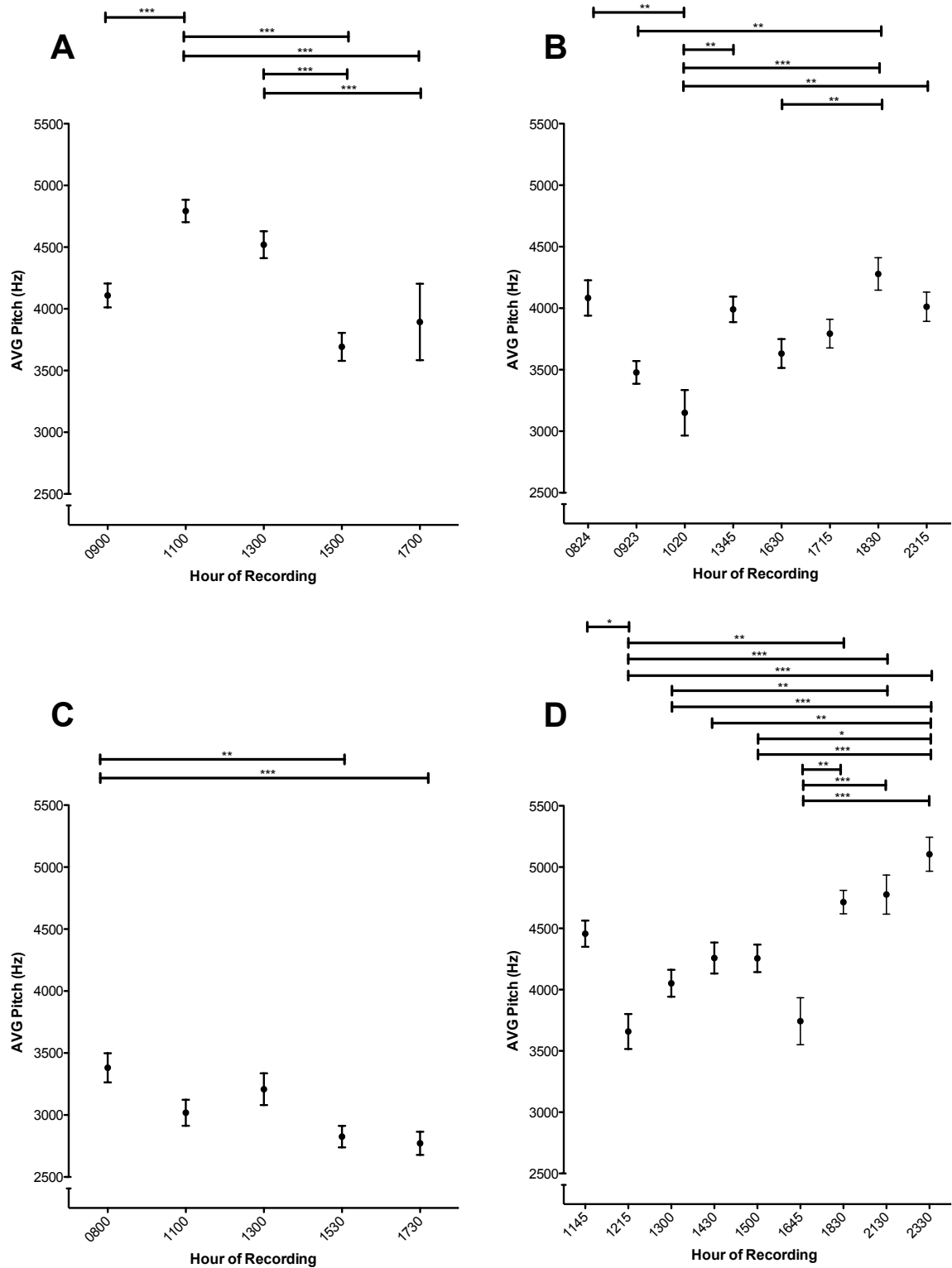
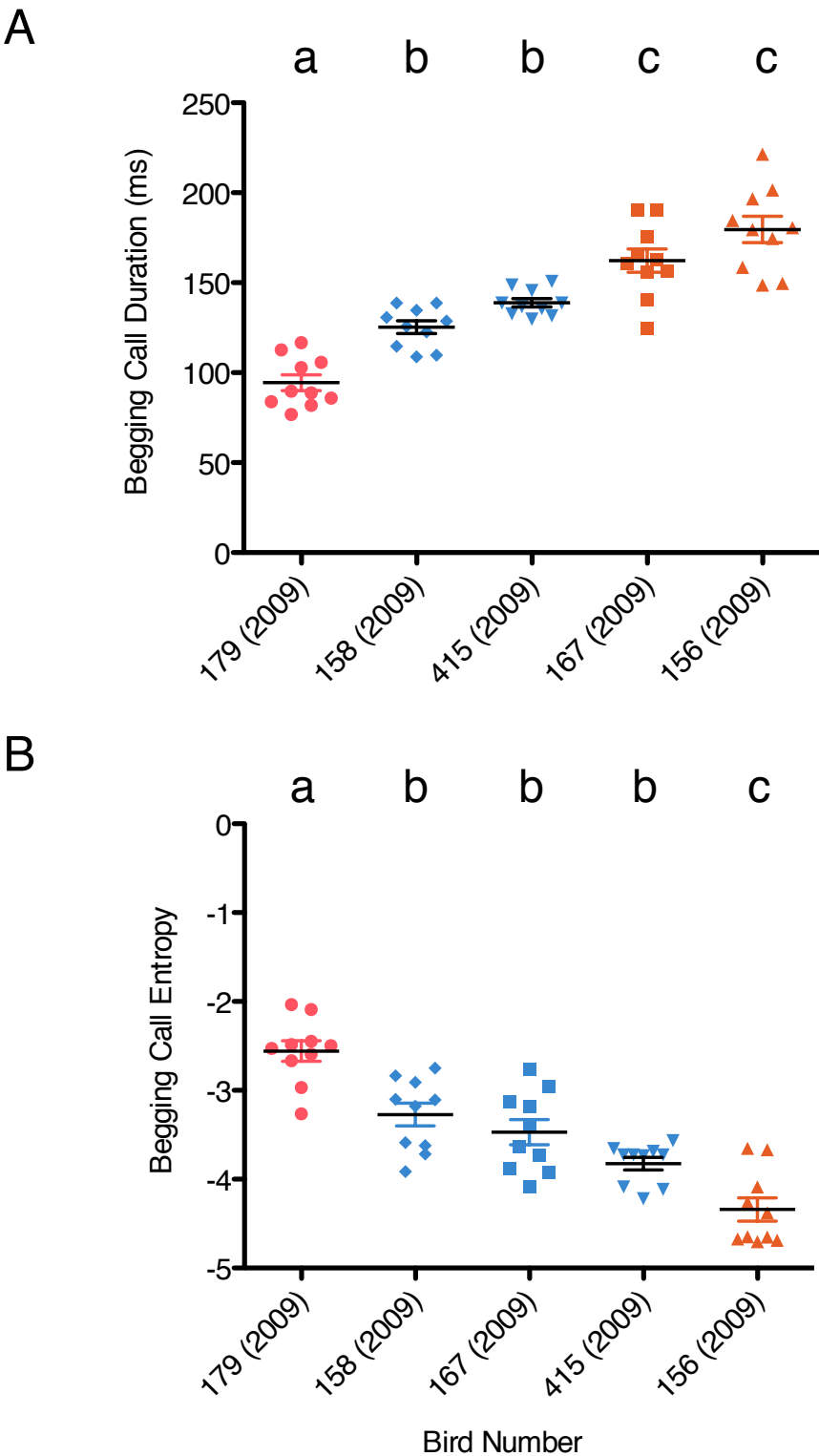


Figure 2.5: The characteristics of food-begging calls significantly differ between individual birds. Ten food-elicited begging calls of five P16 individuals were collected, analyzed and are displayed above. The call duration **(A)** and average call entropy **(B)** are displayed above as representative of differences in call characteristics between birds. Birds are displayed in rank order from shortest to longest average calls **(A)** and most to least average call entropy **(B)** and are thus not necessarily in the same location along the X axis from graph to graph. Birds are color coded whereby similarly colored birds are not statistically different ($p > 0.05$) from each other but are to differently colored birds. The letters (a - c) above the data also reflect these statistical results whereby columns that do not share a letter are significantly different ($\leq p 0.05$). Averages \pm Standard Error shown.

Figure 2.5



Results & conclusions:

Type A food begging calls can significantly differ between birds. A One-way ANOVA revealed significant differences in **call duration** $F(1, 5) = 41.02$, $p < 0.0001$ (Figure 2.5A), **average call entropy** $F(1, 5) = 30.36$, $p < 0.0001$ (Figure 2.5B), **average frequency** $F(1, 5) = 11.60$, $p < 0.0001$ (Data not shown), **average frequency modulation** $F(1, 5) = 13.01$, $p < 0.0001$ (Data not shown), but *not* **average pitch** $F(1, 5) = 27.90$, $p = 0.7126$ (Data not shown). Tukey's post-hoc comparisons revealed significant differences between individual birds (Figure 2.5A, B, data for average frequency and frequency modulation not shown). Duration of food-begging calls in the five P16 males varied significantly, with, for example, one male, 179 (2009), producing calls significantly shorter than all other individuals and almost 50% shorter than the longest call-producing bird. While there is variance in the length of calls produced, note that, for example, 179 (2009) does not produce a single call as long as any calls produced by 156 (2009). The evidence thus suggests that food-begging calls can significantly differ between individual birds of the same age (P16), sex (male) and recorded in a similar context (first feeding in the morning) in a multitude of call characteristics. Intriguingly, previous reports of begging call individuality in songbirds have been able to discriminate nestlings only when using multivariate models incorporating as many as 10 call features while in the present study, almost all call features can differ between birds and utilizing as few as two features can result in a vocal signature. The current data does not allow me to state whether feeding canary parents can discriminate their young, but does

present the most widely divergent begging calls yet described, suggesting this might be possible.

Experiment 3: Can canary parents recognize their young by their A calls?

It is interesting to consider why these canary calls may be so distinctive, and we consider three possibilities. First, the calls, like those of redstarts (**Draganoiu et al., 2006; see Appendix 4**) may convey information that can be used by parents to discriminate young. Generally, offspring recognition by parents is better developed in colonial breeding species, presumably due to selective pressure to exclusively care for one's own young (**Beecher, 1990; Levrero et al., 2012; Levrero et al., 2009**). However, vocal signatures of nestlings has also been described in non-colonial birds (of which canaries are a member) and shown to be used to discriminate individual young (**Draganoiu et al., 2006**). Second, the calls are divergent between individuals but this vocal identity is not used by parents. Thirdly, the individuality of the calls is a byproduct of hand-rearing and does not occur in canary nests. Perhaps, parents actively or passively maintain desired call characteristics via, in a sense, food rewards. For example, we can see that begging baby birds produce calls of widely variable average call durations [see Figure 2.5A, bird 156(2009)]. If it is the case that begging nestling are only fed around an ideal call duration by natural canary parents, say, at 180 \pm 10 ms and nestlings are able to modulate their calls,

nestlings may all soon arrive to produce calls within a small range hovering around 180 ms. Thus, as I feed birds no matter what their call length, or any other feature for that matter, I, in a sense, might allow for greater variability than would be seen if nestlings were raised by 'pickier' canary parents.

In order to directly test if A calls could be used to discriminate nestlings, I sought to assess whether A calls can be used by canary parents to discriminate canary nestlings of one's own nest versus those of another nest. Specifically, I tested if feeding canary parents preferentially respond to the A calls of their offspring versus from those produced by unrelated canary nestlings. To do so, five canary pairs were placed into individual standard breeding cages and allowed to breed, build a nest, lay eggs and incubate them to hatching. The males were permanently removed when the youngest hatchling was between P2 - P4. As often males will aid in the feeding of nestlings either directly or by feeding the mother, three days were allowed for the female to recover from the loss of a feeding partner. All cages that had more than 3 nestlings had the excess nestlings removed and placed in another nest not involved in the current study. All nests now had the same number of nestlings. During the next three days, canary mothers experienced two habituation trials daily to accustom them to later testing conditions. Each habituation trial consisted of two parts. First, the canary cage was covered on all sides, except for the top of the cage and two 'windows' which were placed on opposite ends of the cage (See Figure 2.6 for details) for two hours. Secondly, after one hour of the cage sides being covered, the nest

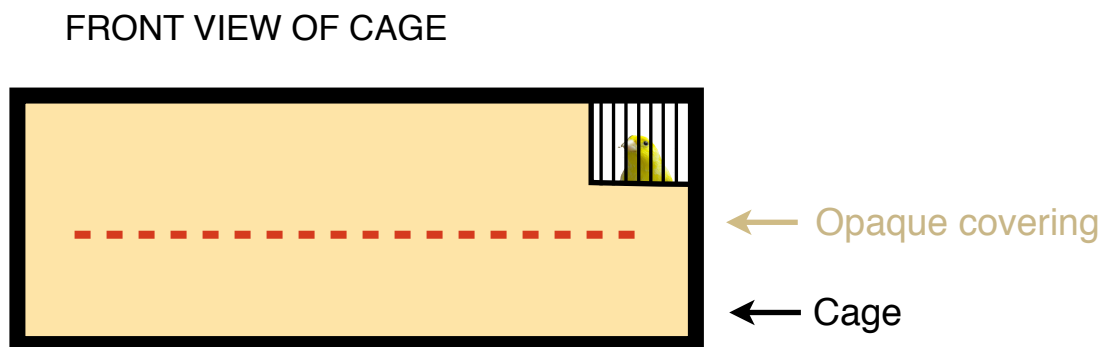
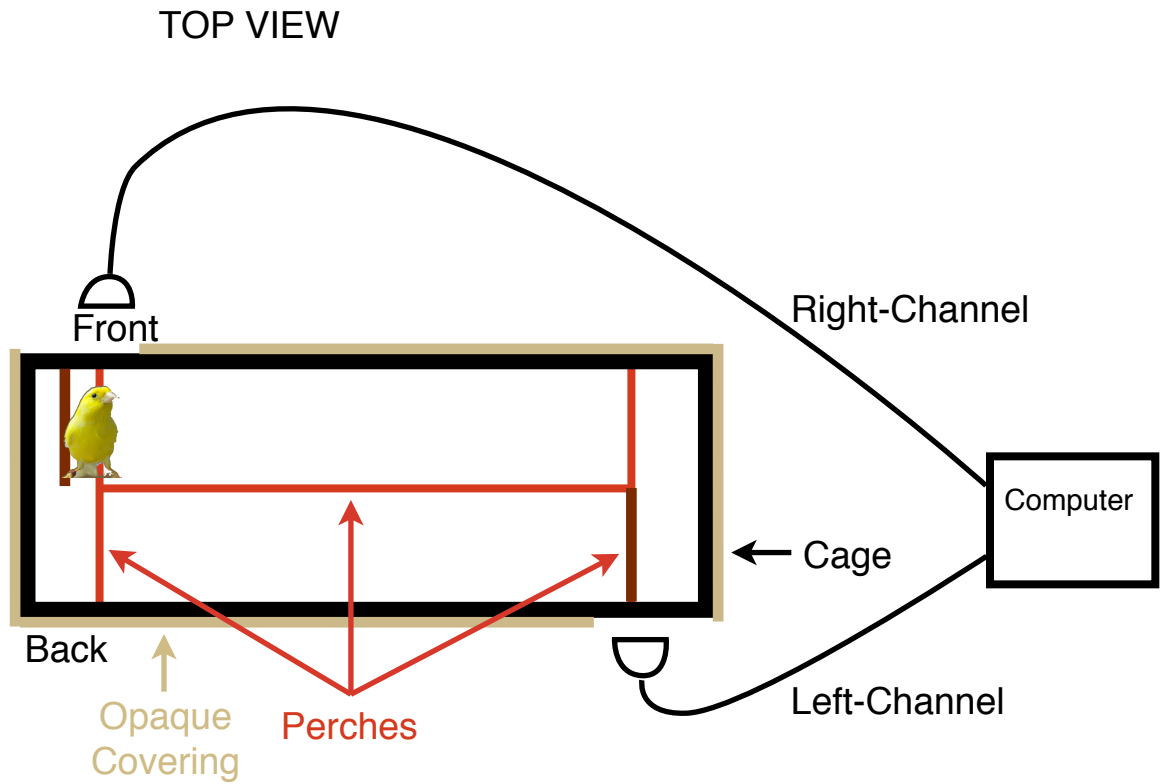
was removed from the cage and placed outside of the cage directly in front of one of the windows. The nest was moved to the opposite window after 15 minutes and this continued for one hour. The side of the cage that received the nest first was counterbalanced from trial to trial. Afterwards, the nest was returned to the cage and the opaque covers removed.

On the third day, after both acclimatization trials, I recorded the begging calls produced by nestlings in each of the five cages. In order to do so, the nest was removed from the cage, taken to a sound proof chamber not in hearing range of the females and kept unfed for 1.5 hours. Afterwards, I elicited begging calls from the nestlings and recorded them using SAP software. At least 8 - 12 seconds of uninterrupted calls were recorded for each nest. Due to the young age of nestlings (Mean age per nest = P11.1), no type B calls were produced. Each nest was then returned to their appropriate cage. Recordings of begging calls for each nest were then trimmed to 6.0 seconds and made to have similar amplitudes using GoldWave software. This length of recording was used because it was the longest recording that would allow every represented nest to have produced a similar number of calls in that time, thus partially controlling for calling rate differences.

On the fourth day, the opaque covers that had been used in habituation trials were added to the cage. After one hour, the nest was removed from inside the cage, taken to a sound proof chamber beyond the hearing of the canary

Figure 2.6: Begging call discrimination assay. (TOP VIEW) A standard flight cage (L: 18 inches, W: 9 inches, H: 10 inches) was slightly modified to include three perches, two side perches and one running perpendicular down the middle. An opaque cover was placed on all sides of the cage except two small port holes that were removed, on opposite corners of the front and back (see Front View) that were aligned with the side perches, thus allowing an animal a 'window' to look out from on the front and back of the cage. The perches nearest each window are colored a darker red only to note where canaries had to be perched to be counted as having approached the begging playback. Audio speakers connected to a laptop were placed ~0.5 m away from each window. Each audio speaker represented either the left or right audio channel. **(FRONT VIEW)** This view nearly identical to the back view and shows the shape of the opaque covering and the underlying cage behind it. A canary is shown in order to depict the level of visual exploration available to an animal tested in the apparatus. The perches are also shown in this view to show their placement height but would naturally be occluded from a frontal view because of the opaque covering. Cage, speakers, perches, etc, not drawn to scale.

Figure 2.6



female, the young fed, and kept there for the remainder of the testing period (~ 1 hour). A speaker connected to a computer was placed ~0.5 m from each of the two windows of the cage containing the female canary being tested. I then sat 4 - 5 meters away from the nest at a slight elevation in order to be able to see into the cage from the top and from there controlled the computer and the sounds played over the speakers (Figure 2.6). All recordings were stripped of identifying information (nest of origin) and given a number. The order of recordings presented were then arranged by another individual. The effect of such controls was to make me blind to which recordings belonged to a particular female. When the female was perched roughly in the middle of the middle perch, begging calls recorded the previous day of either their own young or those of another nest were then played one at a time over one channel, thus directing the vocalizations at one of the two cage windows. The recordings of begging played were 6.0 s and were taken from moments when all of the nestlings were posturing while food begging and thus presumably vocalizing. After 2 minutes, the next time the female was perched in the middle of the center perch, the alternate recording was played over the other speaker directed at the opposite window. This was done a total of 20 times, thus testing the reaction of the female to the begging vocalizations of her own young 10 times and those of another female's 10 times. I scored whether females moved close to the recording-associated window from the central perch in the 10 seconds that followed initiation of the sound recording. To be counted as having moved towards the recording-associated window, females had to perch in the most proximal portion of the perch next to the

window (Dark red portions of the side perches in figure 2.6). The side that produced sound was noted as well as the first window visited -if one was visited at all- during the 10 seconds of response assessment. The percent (%) of times that each canary mother responded to playback of her own nestlings and then of unrelated nestlings was calculated afterwards when the identity of recordings was revealed. The values within each group (playback response to own versus other's young) were compared to each other using a paired samples T-test.

Results & Conclusions:

Female canary mothers preferentially respond to begging call playback of their own nestlings ($M = 54.0\%$, $SD = 19.49\%$) compared to the begging calls of unrelated nestlings ($M = 10.0\%$, $SD = 12.25\%$), $t(5) = 5.047$, $p = 0.0072$ (Figure 2.7).

This is direct evidence that using only begging calls, female canaries can discriminate their own young versus those of others. Furthermore, the data lend some support for the hypothesis that A calls carry structural information that feeding canary parents utilize to identify their own offspring. Of course, there may be other traits that the female canaries cued onto besides the actual calls themselves. For example, differences in the begging intensity, begging call rate, or even the number of begging individuals may all have signaled her own nestlings rather than the structural features of the calls produced. While care was taken to ensure that the recordings were of similar amplitude, presented the

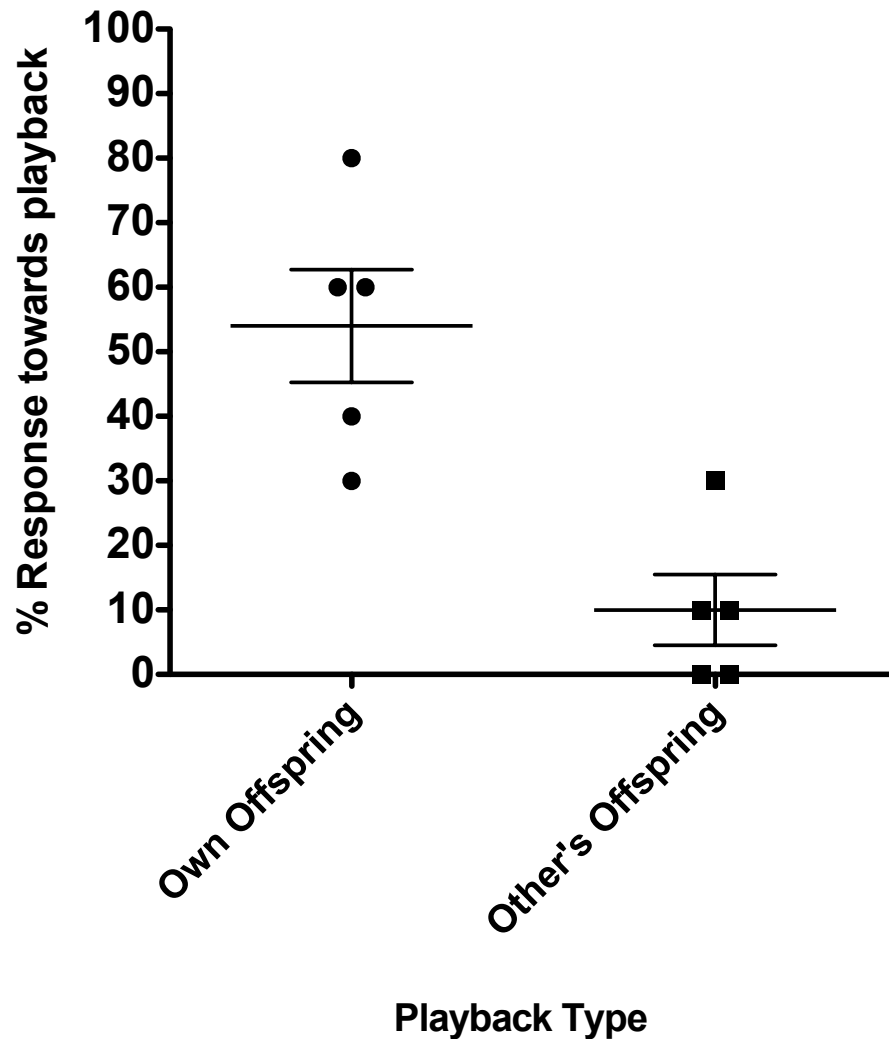


Figure 2.7: Female canaries preferentially respond to playback of their own young's begging calls. The percent of times that a female canary mother responds to playback of her own nestling's A calls is significantly greater than the response to playback of A calls from unrelated young. Every canary mother tested ($n = 5$) responded more to her own offspring.

same number of calls across the recording, and that every nest had the same number of offspring, there may nevertheless be a number of cues that were not controlled for. However, even if canary parents can tell their own offspring apart using call features, we can not know with such an ecologically artificial test whether they actually ever do. Regardless, while the current data does not present direct evidence that adult canaries do discriminate offspring using begging call features, it provides some support for the hypothesis that they can.

Experiment 4: How do begging calls change across development?

As Figure 2.1 clearly shows, a number of changes occur across development in the structure of calls produced during food-begging by one nestling. These calls change from quiet whistles (Figure 2.1, P9 - P12) to the characteristic **A** call which contains 2 elements, a quiet whistle followed by a louder higher frequency inverted-U shaped sound (Figure 2.1, P13 - P17). To more quantitatively understand how food-begging calls change across development, thirty one canaries were recorded daily from P9 - P21 and their **A** calls from each day analyzed using SAP. An average for each bird at every age was calculated for each call feature and averages for each age calculated across birds. Only birds producing at least 100 **type A** begging calls within a day were used in the analysis. As a result, not every bird is represented at every age as a few birds did not beg sufficiently to qualify, and others stopped vocalizing during

food-begging. A One-way ANOVA and Tukey's Multiple Comparison Test were used to test for significance.

Results & conclusions:

While earlier experiments showed great variability in the characteristics of calls within and between individuals, the call features of **type A** calls nevertheless systematically change across development. Due to the multiple comparisons made across different call characteristics, for the features analyzed below, a Bonferroni adjustment was made to the alpha level to protect against Type 1 errors. A new p level of 0.01 ($0.05 / 5$) was set as the significance threshold.

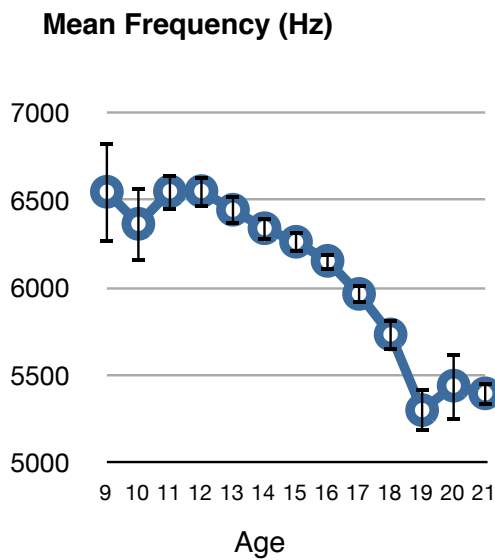
Average Frequency:

A One-Way ANOVA revealed that Mean Frequency changes across development $F(1, 13) = 16.24, p < 0.0001$. Mean **type A** call frequency decreases across development (Figure 2.8, Statistics in Table 2.9).

Average Pitch:

A One-Way ANOVA revealed that mean pitch changes across development $F(1, 13) = 14.90, p < 0.0001$. Mean pitch in **type A** calls decreases across development (Figure 2.10, Statistics in Figure 2.11).

A



B

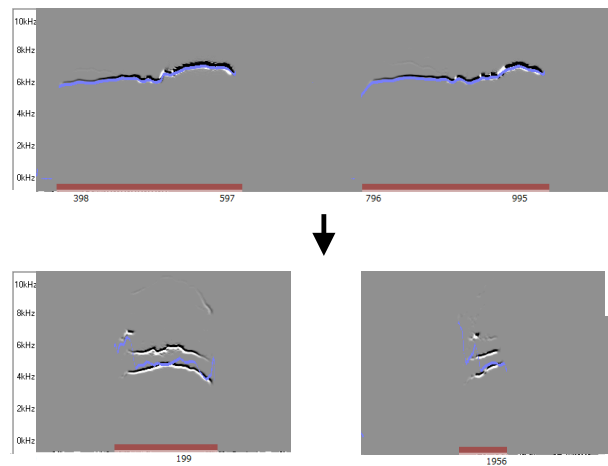
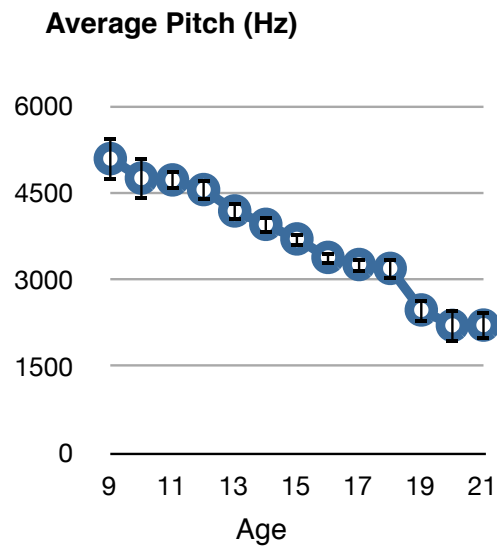


Figure 2.8: Average frequency of type A calls decreases across age. (A) Average call frequency of begging calls across age compiled from 8 - 31 birds per age. Averages \pm Standard Error shown. Statistics are found in the next figure. **(B)** Representative sonograms of A calls (underlined in red) and SAP-produced frequency measurements (blue line) of two calls from one bird at P11 and again at P18. Note the decreased measured frequency at P18, with the blue line at ~4.5KHz from at or above 6KHz at P11.

	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21
P9		ns	ns	ns	ns	ns	ns	ns	ns	*	***	***	***
P10			ns	ns	ns	ns	ns	ns	ns	*	***	**	**
P11				ns	ns	ns	ns	*	***	***	***	***	***
P12					ns	ns	ns	*	***	***	***	***	***
P13						ns	ns	ns	**	***	***	***	***
P14							ns	ns	*	***	***	***	***
P15								ns	ns	***	***	***	***
P16									ns	*	***	**	**
P17										ns	**	ns	ns
P18											ns	ns	ns
P19												ns	ns
P20													ns
P21													

Table 2.9: Statistical comparisons of mean A call frequency from P9 - P21. Statistical post-hoc comparisons between all ages are shown above (refer to Figure 1.8). 8 - 31 birds per group. * = $\leq p$ 0.05, ** = $\leq p$ 0.01, *** = $\leq p$ 0.001.

A



B

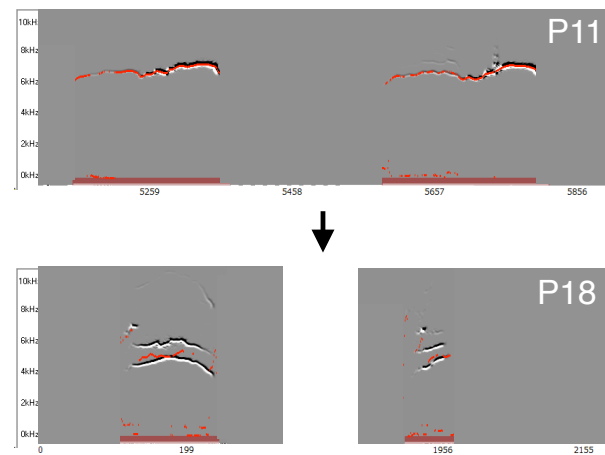


Figure 2.10: Average pitch of type A calls decreases across age. (A) Average pitch of A calls across age compiled from 8 - 31 birds per age. Averages \pm Standard Error shown. Statistics are found in the next figure. **(B)** Representative sonograms of A calls (underlined in red) and SAP-produced pitch measurements (red dots) of two calls from one bird at P11 and again at P18. The SAP-produced dots display SAP measurements of pitch throughout the call. Note that pitch measurements are significantly lower by P18.

	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21
P9		ns	ns	ns	ns	ns	ns	**	**	**	***	***	***
P10			ns	ns	ns	ns	ns	*	**	**	***	***	***
P11				ns	ns	ns	**	***	***	***	***	***	***
P12					ns	ns	*	***	***	***	***	***	***
P13						ns	ns	*	**	**	***	***	***
P14							ns	ns	ns	ns	***	***	***
P15								ns	ns	ns	**	**	**
P16									ns	ns	ns	*	ns
P17										ns	ns	ns	ns
P18											ns	ns	ns
P19												ns	ns
P20													ns
P21													

Table 2.11: Statistical comparisons of average A call pitch from P9 - P21.

Statistical post-hoc comparisons between all ages are shown above (refer to Figure 1.10). 8 - 31 birds per group. * = $\leq p 0.05$, ** = $\leq p 0.01$, *** = $\leq p 0.001$.

Mean Frequency Modulation:

A One-Way ANOVA revealed that mean frequency modulation changes across development $F(1, 13) = 3.979$, $p < 0.0001$ (Figure 2.12). Mean frequency modulation increases across development (Figure 2.12) though Tukey Post-Hoc tests revealed that most day to day comparisons are not significantly different (Table 2.13) due to greater variability within each group (note the F ratio of 3.979).

Mean Entropy:

A One-Way ANOVA revealed that mean entropy changes across development $F(1, 13) = 19.93$, $p < 0.0001$. Mean entropy in **type A** increases across development (Figure 2.14, statistics in Table 2.15).

In conclusion, begging calls are vocalizations that undergo large changes in many structural features across a few days. Intriguingly, as canaries may be able to discriminate young (see experiment 3), significant changes day to day would require parents to track the vocalizations of offspring over time. This might in fact pale in comparison to the potential tracking that must be done of individuals from feeding session to feeding session (Figures 2.3, 2.4). Memory for young is well established in other avian species. For example, King penguins

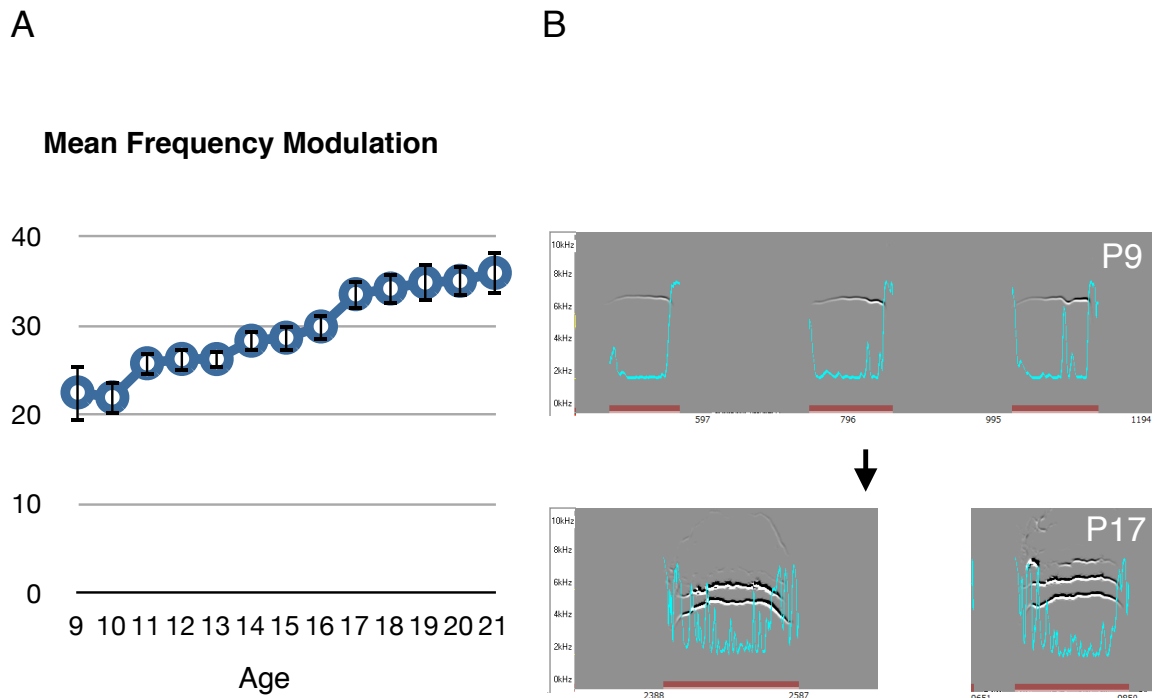


Figure 2.12: Average frequency modulation of type A calls increases across age. **(A)** Average frequency modulation of A calls across age compiled from 8 - 31 birds per are. Averages \pm Standard Error shown. Statistics are found in the next figure. **(B)** Representative sonograms of begging calls (underlined in red) and SAP-produced frequency modulation measurements (turquoise line) of three calls from one bird at P9 and two calls at P17. The SAP-produced line displays changes in frequency whereby flat lines reflect no change in frequency and vertical lines measure the extent of frequency changes. For example, at P9, when the bird produces close to a pure-tone whistle, there are few changes in frequency but, by P17, there are greater and more frequent frequency changes throughout the call.

	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21
P9		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
P10			ns	ns	ns	ns	ns	ns	*	*	*	ns	ns
P11				ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
P12					ns	ns	ns	ns	ns	ns	ns	ns	ns
P13						ns	ns	ns	ns	ns	ns	ns	ns
P14							ns	ns	ns	ns	ns	ns	ns
P15								ns	ns	ns	ns	ns	ns
P16									ns	ns	ns	ns	ns
P17										ns	ns	ns	ns
P18											ns	ns	ns
P19												ns	ns
P20													ns
P21													

Table 2.13: Statistical comparisons of average A call frequency modulation from P9 - P21. Statistical post-hoc comparisons between all ages are shown above (refer to Figure 1.12). 8 - 31 birds per group. * = $\leq p$ 0.05.

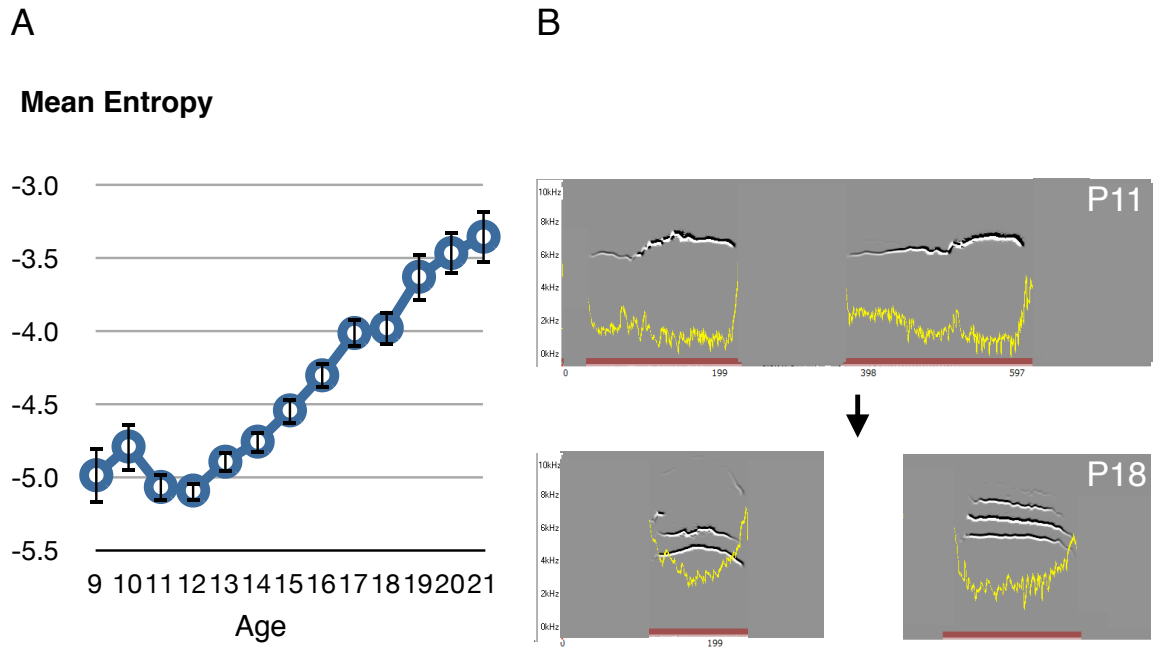


Figure 2.14: Average entropy of type A calls increases across age. (A) Average entropy of A calls across age compiled from 8 - 31 birds per time-point. Averages \pm Standard Error shown. Statistics are found in the next figure. **(B)** Representative sonograms of A calls (underlined in red) and SAP-produced entropy measurements (yellow line) of two calls from one bird at P11 and again at P18. Note the higher entropy measurements throughout the call by P18.

	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21
P9		ns	ns	ns	ns	ns	ns	ns	*	*	***	***	***
P10			ns	ns	ns	ns	ns	ns	*	*	***	***	***
P11				ns	ns	ns	*	***	***	***	***	***	***
P12					ns	ns	**	***	***	***	***	***	***
P13						ns	ns	**	***	***	***	***	***
P14							ns	ns	***	***	***	***	***
P15								ns	**	**	***	***	***
P16									ns	ns	**	**	**
P17										ns	ns	ns	ns
P18											ns	ns	ns
P19												ns	ns
P20													ns
P21													

Table 2.15: Statistical comparisons of average **A call entropy from P9 - P21.** Statistical post-hoc comparisons between all ages are shown above (refer to Figure 1.14). 8 - 31 birds per group. * = $\leq p$ 0.05, ** = $\leq p$ 0.01, *** = $\leq p$ 0.001.

locate their young amongst thousands of chicks after months of separation (Aubin & Jouventin, 1998)⁷. What neural circuits might be involved in the memory of young, whether long or short term, is unknown. Functionally however, stereotyped changes in call structure over time such as decreases in mean frequency and increased entropy might allow feeding canary parents to discriminate young based on age, as has been shown in a number of species (Saino et al., 2000; Smiseth, Amundsen, & Hansen, 1998; Teather, 1992).

Experiment 5: Are begging calls sexually dimorphic?

The studies thus far presented have given clear evidence for the dynamic nature of A call structure within and across days. The work has also documented a number of features that might theoretically be taken advantage of to discriminate young, which canary parents appear able to do. Sexual dimorphism may also be such a feature as a number of studies have shown that some bird species have dimorphic food-begging calls (M.E. Hauber & C.K. Ramsey, 2003; Liu et al., 2009; Saino et al., 2003; Saino et al., 2008). To test if **type A** food-begging calls in the canary are sexually dimorphic, how they might be different, and, critically, when these differences might arise, the data of 11 males and 11 canary female nestlings from experiment 2 were segregated and their data reaveraged. A Two-way between groups analysis of variance was conducted to

⁷ Mother northern fur seals -notably, not birds- have also been shown to respond to the call of their pups even after 4 years of separation! Insley SJ (2000) Nature 406: 404-405.

explore the impact of sex and age on **type A** begging call pitch, duration, frequency modulation and entropy.

Average Pitch (Figure 2.16A):

As expected from experiment 2, there was a statistically significant main effect for age, $F(2, 9) = 67.26$, $p = <0.0001$. Additionally, there was also a statistically significant main effect for sex, $F(0, 1) = 15.36$, $p = 0.0009$, and a statistically significant interaction between age and sex, $F(2, 9) = 2.839$, $p = <0.004$. Post-Hoc comparisons revealed that pitch was significantly higher in females than in males at P12 ($p < 0.05$), P19 ($p < 0.001$) and P20 ($p < 0.001$; Figure 2.16A).

Average Call Duration (Figure 2.16B):

There was a statistically significant main effect for age, $F(2, 9) = 3.352$, $p = 0.0008$. There was no main effect for sex, $F(0, 1) = 0.15$, $p = 0.24$. Thus, no Tukey post-hoc test was performed. There was however a statistically significant interaction between age and sex, $F(2, 9) = 5.233$, $p = <0.0001$.

Average Entropy (Figure 2.16C):

There was a statistically significant main effect for age, $F(2, 9) = 69.21$, $p = <0.0001$. There was also a statistically significant main effect for sex, $F(0, 1) = 8.174$, $p = 0.0097$, and a significant interaction between age and sex, $F(2, 9) = 5.955$, $p = <0.0001$. The female calls were less “noisy,” a difference that increased with age. Post-Hoc comparisons

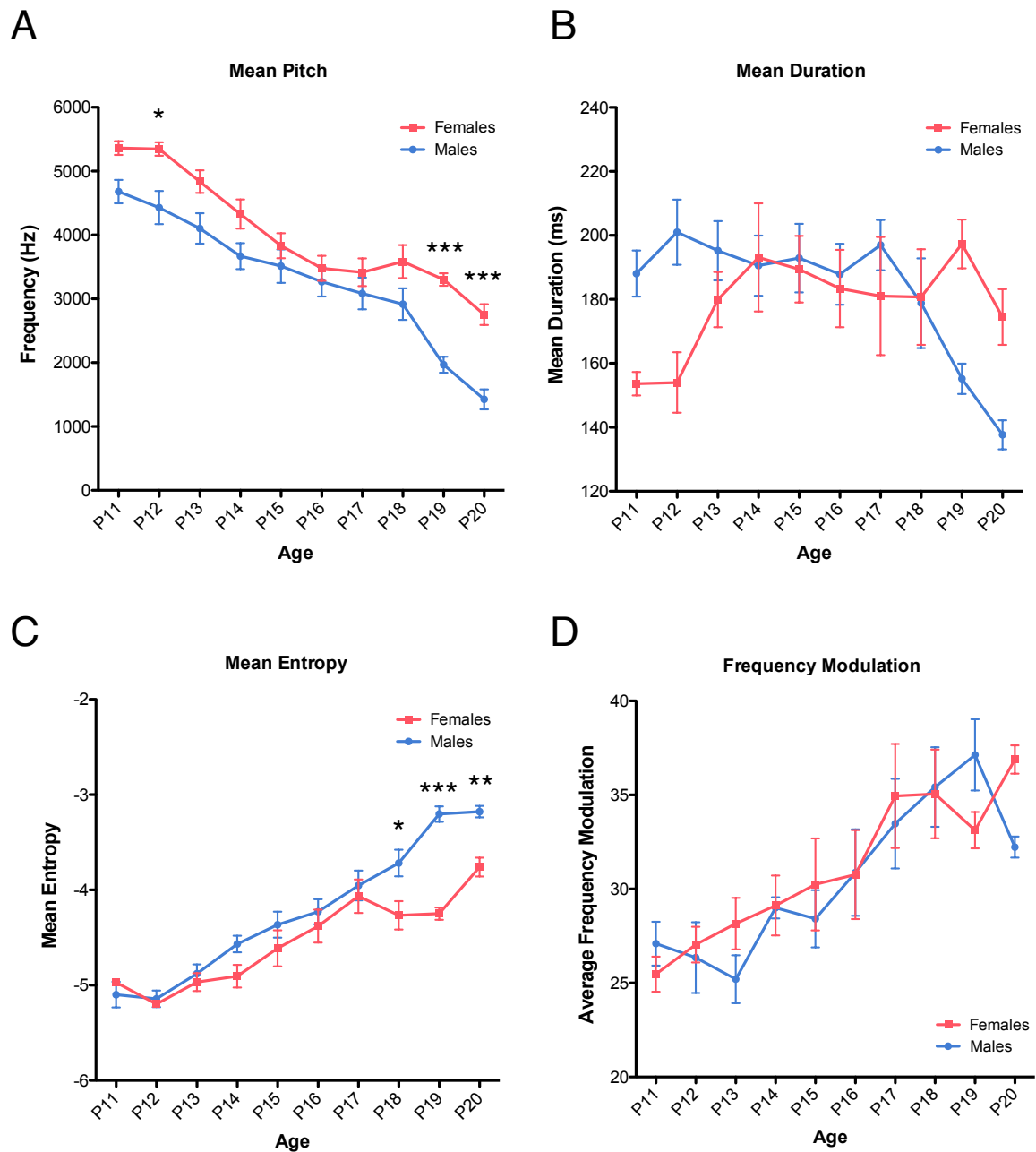


Figure 2.16: Type A begging calls are sexually dimorphic in some but not all call features. * = $\leq p$ 0.05, ** = $\leq p$ 0.01, * = $\leq p$ 0.001.**

revealed that entropy was significantly different between the sexes at P18 ($p < 0.05$), P19 ($p < 0.001$) and P20 ($p < 0.01$).

Average Frequency Modulation (Figure 2.16D):

As predicted from experiment 2, there was a statistically significant main effect for age, $F(2, 9) = 12.27$, $p = <0.0001$. There was, however, neither a statistically significant main effect for sex, $F(0, 1) = 0.15$, $p = 0.70$ nor a statistically significant interaction between age and sex, $F(2, 9) = 1.29$, $p = 0.24$.

Sexual dimorphism in food-begging calls has been described late in fledgling development (P16 and later) in a number of other species including barn swallows (Saino et al., 2003; Saino et al., 2008), catbirds (M.E. Hauber & C.K. Ramsey, 2003; see Appendix 4), and chipping sparrows (Liu et al., 2009). However, I here present evidence that sexual dimorphism in food-begging calls may be present from very early in development (Figure 2.16A) and can be found in some, though not all, call features. There are two particularly interesting aspect to these results. First, frequency modulation is yet another feature that can be used to discriminate nestlings, suggesting that there are ample signals in food begging calls to discriminate individuals. Secondly, sexual dimorphism in begging calls appeared in more features later in begging ontogeny, supporting numerous previous findings that later begging calls can be sexually dimorphic (M. E. Hauber & C.K. Ramsey, 2003; Liu et al., 2009; Saino et al., 2003; Saino et al., 2008). The reason for this widening dimorphism at later ages may be related to

forebrain activity, as begging activity increases c-fos expression in RA in older male chicks and RA lesions affect the variability of begging calls in male, but not female, chirping sparrows (Liu et al., 2009). Whatever the mechanisms, some of these structural differences in the call appear late in ontogeny, highlighting a difference between early and late begging calls.

Experiment 6: Are **B** calls a *structurally* distinct call type?

Type **B** calls of canary nestlings are identified by the characteristic rapid frequency undulations (Figure 2.17). Though the sound and spectrographic morphology of **B** calls is unmistakably distinct to a trained observer and thus differences in call characteristics between **A** and **B** calls are expected, I sought to analyze specifically how **B** calls were structurally different from **A** calls. To do so, 10 **A** and 10 **B** calls from five P17 individuals were analyzed for call characteristics using SAP. Averages for each call type for every individual were calculated and scores for **A** and **B** calls compared to one another using an independent-samples t-test.

Results & Conclusions:

A calls ($M = 140.1$ ms, $SD = 32.99$ ms) are significantly **shorter** than **B** calls ($M = 242.9$ ms, $SD = 76.82$ ms), $t(4) = 5.421$, $p = 0.0251$ (Figure 2.18).

A calls ($M = -4.360$, $SD = 0.4042$) have significantly **lower average entropy** than **B** calls ($M = -3.493$, $SD = 0.6619$), $t(4) = 2.681$, $p = 0.0369$ (Figure 2.18).

P18 M

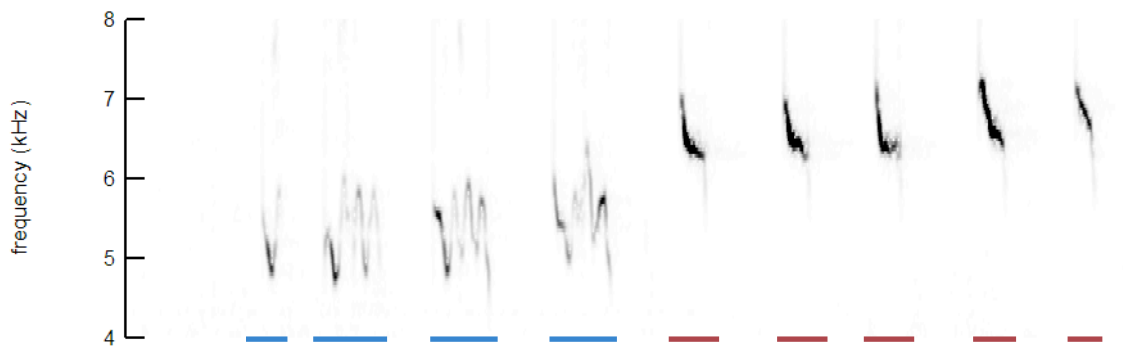
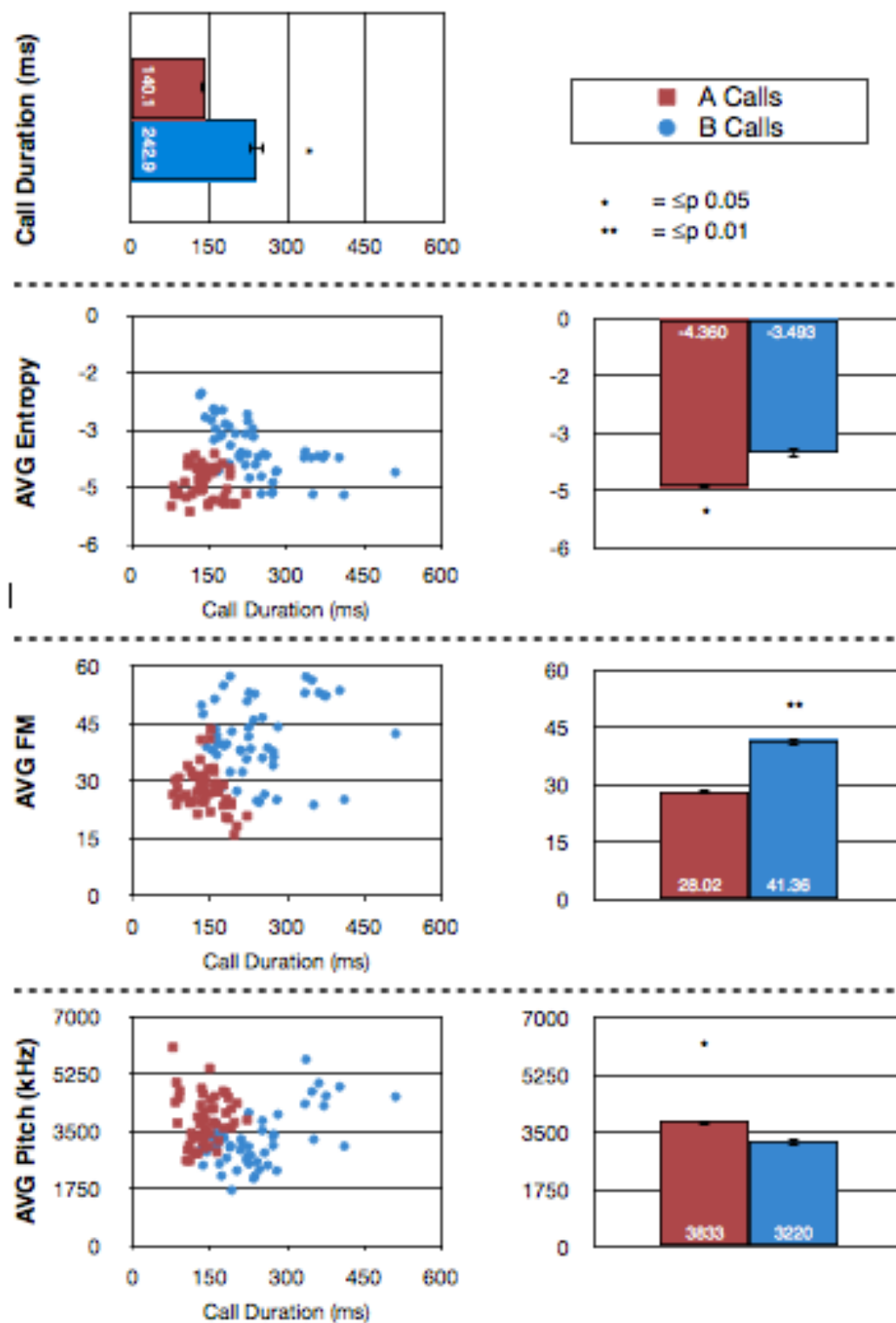


Figure 2.17: Type A and B calls appear to be structurally distinct call types. The food-elicited calls produced in one continuous recording of one P18 male are displayed above. B calls are underlined in blue and A calls are underlined in red. Note that B calls are of lower frequency than A calls and are characterized by higher amounts of frequency modulation.

Figure 2.18: A and B calls are structurally distinct call features. Ten A calls and ten B calls for each of five P17 birds were analyzed for features using SAP. Scatter plots do not differentiate individual birds, just call type. Within each bird, an average was taken for each feature and birds averaged to produce the results shown in bar graphs. A and B calls were significantly different for every animal for every feature shown above. Average call duration, entropy, frequency modulation, and pitch were significantly different between the two call types. B calls are longer, are more modulated, have higher entropy and are on average produced at a lower pitch than A calls.

Figure 2.18



A calls ($M = 3833$ Hz, $SD = 503.0$ Hz) have significantly **higher average pitch** than **B** calls ($M = 3220$ Hz, $SD = 791.6$ Hz), $t(4) = 1.259$, $p = 0.0200$ (Figure 2.18).

A calls ($M = 28.02$, $SD = 4.463$) have significantly **lower average frequency modulation** than **B** calls ($M = 41.36$, $SD = 7.251$), $t(4) = 2.640$, $p = 0.0080$ (Figure 2.18).

A calls ($M = 2767$ Hz, $SD = 559.1$ Hz) have a significantly **smaller frequency range** than **B** calls ($M = 4444$ Hz, $SD = 291.0$ Hz), $t(4) = 3.691$, $p = 0.0003$ (Data not shown).

The design features of calls, presumably shaped by evolutionary forces, may give insight into their communicative purpose. Calls with wide frequency range, abrupt onset and/or termination, and modulations of frequency, all allow birds and mammals to more easily locate the source in 3D space, while high pitch sounds, pure tones, absence of frequency modulation and smooth gradients of amplitude at the onset or termination of the call make locating their source position difficult (Brown, 1982; Klump & Shalter, 1984; Marler, 1955; Redondo & Arias De Reyna, 1988; Rooke & Knight, 1977). In fact, we see these very differences in the structural design between **A** and **B** calls; **A** calls have a smaller frequency range (~ 2.8 kHz) than **B** calls (~ 4.4 kHz), a higher pitch (Figure 2.19), less frequency modulation (Figure 2.19), and only the onset of **A** calls is marked by a lower amplitude element that gives rise to a higher amplitude one (Figure 2.2). Thus, the differences in structure of the two calls suggest

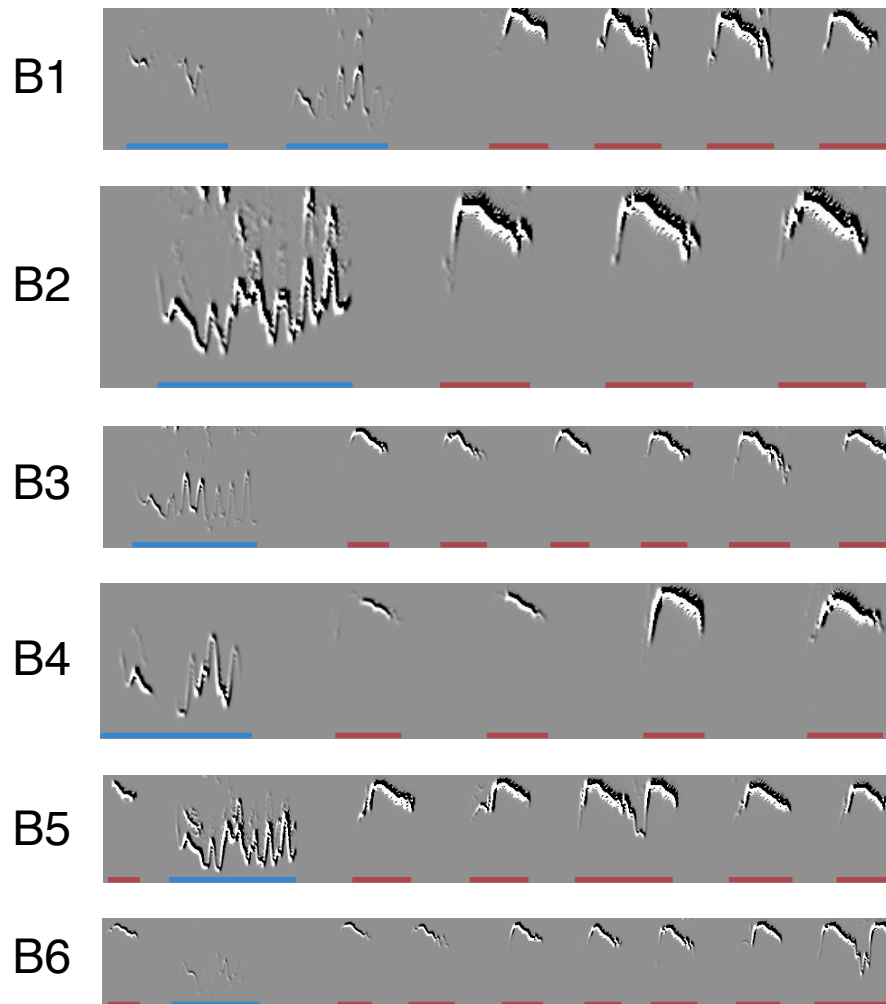


Figure 2.19: Type B calls appear to be produced at the beginning of begging bouts. Six consecutive begging bouts during one feeding session from one P17 male canary. B calls are underlined in blue and A calls are underlined in red. Note that sonograms have been enlarged or shrunk to better display the entire begging bout and are thus *not* displaying equal amounts of time on the X-axis.

potential for differential communicative roles: **A** calls appear structurally suited to obscure position but nevertheless provide an auditory signal of nutritional need, while **B** calls are particularly well-suited for communicating source position. A vocal signal in birds that has a well-established role in communicating location of source in songbirds are contact calls. Contact calls are generally highly frequency modulated calls produced by birds visually separated, often during foraging, in order to keep contact and maintain group proximity (46, 47, 48). Whether **B** calls may play a functionally similar role to contact calls in signaling location in nestling canaries can not yet be established, no matter how suggestive of function the structural design of **B** calls might be, and was the next subject of study.

Experiment 7: Are **B calls a *functionally* distinct call type?**

As noted in Figure 2.1, **B** calls appear later in development than **A** calls. I next sought to understand whether **B** calls were indeed a functionally distinct call type - perhaps serving a contact call role- or whether this was the first indication of further modifications to the begging call as had occurred throughout development (Figure 2.1).

Study 1

I began by assessing whether **type B** calls were produced in distinct contexts. First, I tested whether these calls were produced during food-begging,

i.e. whether they were elicited after food deprivation by food presented within ~5 cm from beak, as the bird stretched its neck, gaped, and beat its wings. Fifteen canary fledglings (P18 - P20) that were identified as producing **type B** calls were video recorded during a feeding session. The video was then scored for whether the bird produced **type B** calls during food-begging as well as whether the bird produced typical **type A** calls during food-begging. Videos were scored by an individual blind to the hypotheses, age of the birds, or treatments. 'Yes' or 'No' was noted for each call type within each bird and chi-square goodness-of-fit test was done to determine if the calls were present or absent during food-begging.

Study 1 Results & Conclusions:

Thirteen birds of the fifteen tested produced **type B** calls during video recordings of food-begging. A chi-square goodness-of-fit analysis indicated that there was no significant difference in the proportion of birds identified in the current sample that produced **type B** calls during food-begging (86.7%) as compared to those that produced **type A** calls during food-begging (100%), $X^2 (1, n = 15) = 2.143, p = 0.1432$. Thus, birds appear to use the **B** call, like the **A** call during food-begging. However, their presence while food begging is under way does not mean that the call is used to communicate the same information as the **A** call. Of course, the present study does not allow us to assess the communicative role of **B** calls or how they might differ from **A** calls.

Study 2

I had noticed while recording birds that B calls were occasionally produced without posturing, something that I had not witnessed with type A food-begging calls, as these calls are almost always produced with an open beak and while physically posturing (Though posture extent can vary widely; Kedar, Rodriguez-Girones, Yedvab, Winkler, & Lotem, 2000). I thus sought to more rigorously test whether B calls could be used in a *different* context than type A calls. I video recorded thirteen fledgling canaries (P16-P20) that had been identified as producing B calls. The fledglings were food deprived for 2.5 hours (feedings typically take place every 1.5-2 hours) ensuring that they would be hungry. The investigator, which was not seen for the 2.5 hours prior but was historically always the one to feed birds and thus was associated with food, approached the nest containing the fledglings but stopped 4 feet away and presented no food. After one minute, the investigator approached the fledgling, presented food, and fed the animal. Video analysis was conducted by an investigator blind to the experimental conditions and hypotheses. Videos were scored for whether A or B calls were produced *without* posturing while the investigator stood 4 feet away. For these studies, the presence of any posturing behavior (wing flapping, extended neck, open beak) was scored as posturing. A 'Yes' or 'No' was marked for each of the two call types. A chi-square goodness-of-fit test was conducted to test whether type A or B calls are produced in different contexts.

Study 2 Results & Conclusions:

Of the 13 fledglings tested (as stated earlier, ages P18-P20) 10 produced **B** calls *without* posturing and 0 produced the **type A** calls *without* posturing. The chi-square goodness-of-fit test indicated a significant difference in the proportion of fledglings that produced the **type B** calls without posturing (76.9%) and the proportion of fledglings that produced the typical **type A** food-begging call without posturing (0%), $X^2 (1, n = 13) = 16.25, p < 0.0001$. Thus, the **type B** call can be produced without posturing, a context that is different from the **A** call.

Furthermore, this is the first evidence presented here that **B** calls are a distinct call type not only in acoustic features but to some extent, too, in the contexts they are used. That **B** calls were produced when the ‘feeding parent’ was 4 feet away, a distance not close enough to directly feed, and that these calls were made without the posturing that is associated with food begging (wings flapping, neck extended) and the receiving of food (beak agape), suggests a different communicative role for **B** calls.

Study 3

In other bird species, contact calls are used to maintain contact between visually separated individuals (Bradbury, 2003; Farabaugh & Dooling, 1996; Forshaw, 1989). To continue studying whether **B** calls might serve a contact-call-like function, I next tested whether either call was produced when the animal was visually isolated. Eleven canary fledglings (P17 - P21) that had been identified as producing **B** calls were isolated from nest mates and visually isolated behind an

opaque wall where each bird was by itself and could not see others but could hear them. A microphone was placed near the nest and the animal was then food deprived for three hours (feedings predominantly occur every 1.5 - 3 hours) to ensure the animal was hungry and would vocalize as otherwise fledglings are largely quiet. The entire experiment occurred between 1100 and 1400 hours. Sound recordings of the last hour of visual isolation were then analyzed for the presence of type A or B calls by an investigator blind to the treatments or hypotheses. A 'Yes' or 'No' was noted for each call type for every bird. A chi-square goodness-of-fit test was done to determine if the calls were differentially produced in visually isolated birds.

Study 3 Results & Conclusions:

Eight birds of the eleven tested produced type B calls while visually isolated. In contrast, only one of the eleven birds produced type A calls. A chi-square goodness-of-fit analysis indicated that there was a significant difference in the proportion of birds that produced type B calls during visual isolation (72.7%) compared to those that produced type A calls (9.1%), $X^2 (1, n = 11) = 6.600, p = 0.0024$. Thus, birds that are hungry and visually isolated are more likely to use B calls than A calls. Furthermore, their production during visual isolation, coupled with their characteristics for locatability, support a role as contact-like calls.

Caveat

The presumably increased hunger state of the animals in this experiment must be considered. Food deprivation of 3 hours was used to induce vocalizations in otherwise quiet nestlings and may cloud how we interpret the results above. Perhaps B calls are just food begging calls and thus purely reflect the hunger state of the animal. If this were the case however, A calls would have been similarly produced. They were not. Thus, while the data do not support the interpretation that A and B calls are interchangeable in a visually isolated setting, the hunger state of the animal does not allow us to conclude that these calls were purely contact calls as B calls might, for example, be long-distance food begging calls. For example, the choice of which call to produce (A vs B) might be triggered by parental proximity. If the bird is hungry and the parent is close, the bird produces an A call. If the bird is hungry and the parent is not close or is out of sight, it produces a B call. In both cases, the calls are triggered by hunger state but the context determines the choice of call. Even while work presented earlier showed that these calls can be produced without posturing, we can not assume that all food-begging is done *while* posturing. In conclusion, B calls are used in contexts similar to contact calls and have structural features that make them well-suited to be contact-like calls, but we can not yet rule out a food-begging role for them. Indeed, it might be most appropriate considering all the work thus far presented showing their role in both feeding and visual isolation contexts to call them contact begging calls.

Study 4

We next considered that perhaps our initial study on the presence of **B** calls during food begging was too coarse and that we may have missed a pattern to the production of these calls during food begging that might provide insights into their role within that context. We hypothesized that if indeed **B** calls are distinct in function or communicative value, it might appear at distinct points within a begging bout, much like a capital letter marks the beginning of a sentence or a period the end of one. To test whether **B** calls are found at specific points along the begging bout, I video recorded nine fledglings (P16-P18) that were identified to produce **B** calls and scored every vocalization as type **A** or **B** at every call position in each begging bout (For example, see Figure 2.19). The percent of calls that were **B** calls was calculated for the first five call positions in a bout. A One-Way ANOVA and Tukey's Post-hoc test was carried out to test whether **type B** calls were preferentially produced from the first to the fifth call position in the bout.

Study 4 Results & Conclusions:

A One-Way ANOVA revealed a significant effect at a $p < 0.05$ level of **type B** call preponderance at begging bout position. A Tukey's Post-Hoc test revealed that **B** calls were produced at a significantly higher rate at the first begging position than the fourth or fifth (Figure 2.20). Thus, **B** calls are preferentially produced at the beginning of begging bouts, suggesting a distinct role while food begging. We might hypothesize that within the food begging context, their role is

to signal location, attract attention and/or communicate nestling identity before producing the “I’m hungry/please feed me” **A** call (M.E. Hauber & C.K. Ramsey, 2003; M.L. Leonard & A.G. Horn, 2001). However, while we do not here present data to better define the role of **B** calls, our data nevertheless suggests that **B** calls are not used interchangeably with **A** calls during food begging. The potential for a secondary call type of different communicative function during food begging besides the well-established role of **A**-like calls to signal hunger state (H. C. J. Godfray, 1991; R. M. Kilner & N. B. Davies, 1999; Kilner et al., 1999; Lessells et al., 2011; Price et al., 1996) has not been incorporated into many signaling theories (H. C. Godfray, 1995; H. C. J. Godfray & R. A. Johnstone, 2000; Kilner & Johnstone, 1997) and would necessarily increase the complexity of parent-offspring evolutionary strategies. For example, if both calls induce feeding and **A** calls are an honest signal of nutritional need, and **B** calls are not, fledglings may be under evolutionary pressure to disguise a lack of nutritional need and produce a greater percentage of **B** calls to gain additional feedings. Parents for their part might bias feedings towards birds producing more honest signals in order to more fairly distribute scarce resources.

Experiment 8: When do **B calls appear in development?**

I had noticed, in a cursory manner, that **B** calls appeared later in development than **A** calls (for example, see figure 2.1) but had no quantitative information on this point. To better understand when these calls first emerged,

seventy canary nestlings (P13-P17) were video and audio recorded and their food-begging calls analyzed for call type (A vs B) by an investigator blind to the age of the birds or hypotheses of the experiment. Each animal was recorded only once in the whole experiment, across one feeding session. The percent of B calls at the first position in begging bouts was calculated for each animal and plotted per age. A One-Way ANOVA and a Tukey's Post-hoc test were carried out to test for statistical significance in the differences in incidence of B calls with age.

A note:

There were two reasons that only the first call in begging bouts was analyzed. Firstly, and critically, using all the calls produced during food-begging did not change the results presented here. Secondly, as B calls are more often produced near the front of begging bouts (Figure 2.20), looking at only the first call allowed me to eliminate the variance of scores between birds that arises when different birds produce more calls. For example, if one individual produces 1 call and another produces 100, but each produces a B call only at the first position, the percentage of B calls produced would be 100% and 1% respectively, inappropriately skewing the data for my purposes.

Results & Conclusions:

A One-Way ANOVA revealed an effect of age on the percent of B calls produced, $F(1, 69) = 8.327$, $p < 0.001$. A Tukey's Post-Hoc test revealed that P12-P15 were statistically similar and P16 and P17 were as well. However, all

P12-P15 ages were significantly different from P16 and P17 (Figure 2.21, statistics in Table 2.22). Specifically, no birds were identified younger than P16 days of age that produced a single B call in the feeding sessions recorded. It is

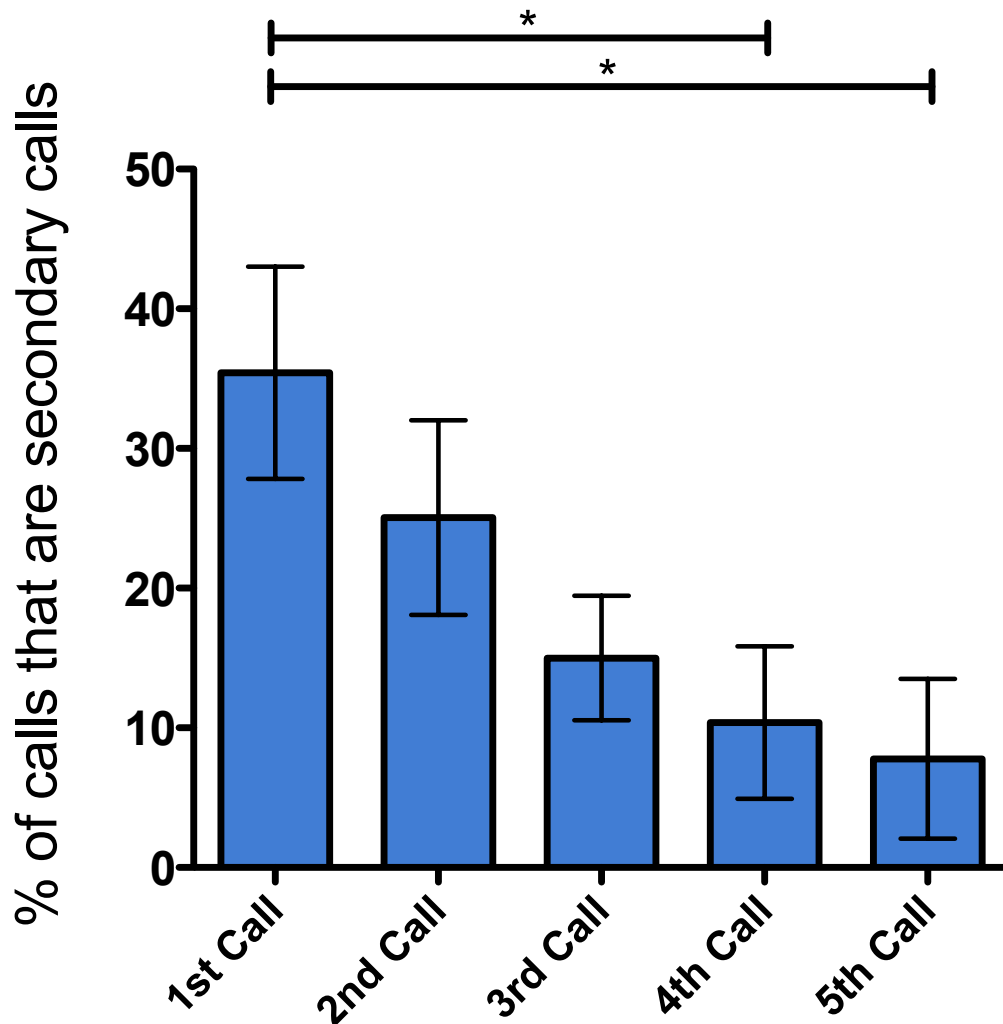


Figure 2.20: Type B calls are predominantly produced at the beginning of a begging bout. Nine fledglings (P16-P18) were video recorded and the call type produced at each position of a begging bout was noted. Shown above are the percentage of calls produced that were B calls as each of the first five vocalizations. Averages \pm Standard Error shown. * = $\leq p$ 0.05

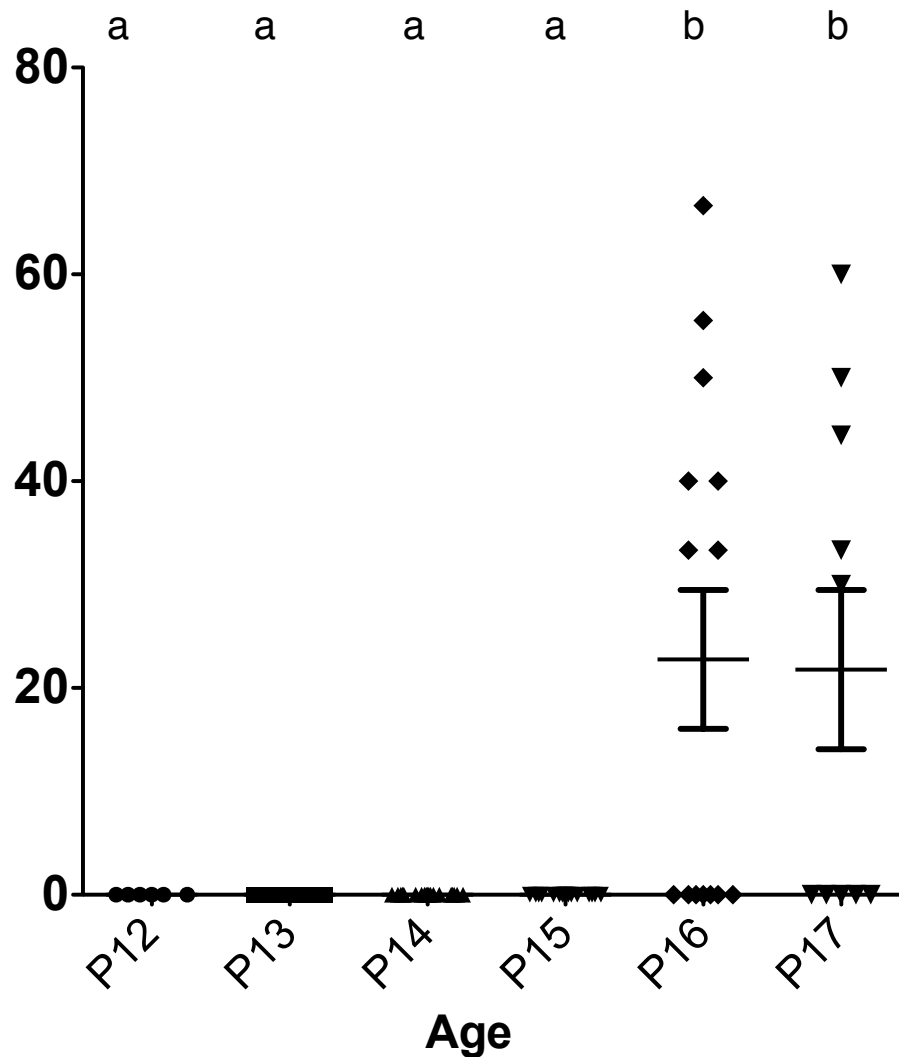


Figure 2.21: Type B calls are first observed at P16. Seventy instances of birds between P12 - P17 were observed and scored for type A and B calls. The percent of all calls produced that were B calls for every animal is displayed above. Averages \pm Standard Error shown. Beginning at P16, but not before, a subset of birds began producing B calls. There were no sex differences in which birds produced or did not produce B calls. Columns that do not share a letter are significantly different ($\leq p$ 0.05).

	P12	P13	P14	P15	P16	P17
P12		ns	ns	ns	*	*
P13			ns	ns	**	*
P14				ns	***	**
P15					***	**
P16						ns
P17						

Table 2.22: Statistical comparisons of percent of B calls produced at each age. * = $\leq p$ 0.05, ** = $\leq p$ 0.01, * = $\leq p$ 0.001.**

worth noting as well that not all nestlings produced B calls at P16 or P17. Thus, while not all birds produce B calls, only birds P16 and older do.

Caveat

It is important to note that I select B calls by one main call feature, their frequency undulations. Thus, there might be B calls that are not sufficiently B-like as determined by the quantifying investigator and so are not sorted appropriately. This would lead to an underrepresentation of B calls in any particular analysis. What I'm arguing in essence is that there might be nuance that I have missed. While true, the contextual differences of B calls as I've defined them nevertheless strongly suggest that type A and B calls are distinct signals.

Experiment 9: Why do B calls arise?

The experiments I have presented thus far have given weight to the hypothesis that B calls may serve a separate communicative function than A calls. B calls are structurally unique calls that are preferentially produced by hungry juveniles in visual isolation. Furthermore, B calls have the structural features to better aid source location and thus their use in isolation settings makes sense if the goal is to be located. Lastly, B calls first arise in our canary nestlings at P16. It was this last piece of unexpected evidence that finally led me to ask why these calls might arise in the first place. If B calls do indeed serve the new function of communicating location to parents for continued feeding -as B

calls are still produced in food begging contexts- then it suggested that the birds would *need* to be located. While my birds were always artificially close to the nest because of the cage arrangement I raised my nestlings in, this is not so in the wild where birds leave the nest (called fledging) before parental care has ended to allow for parents to begin laying and incubating a new clutch of eggs. Thus, I next sought to determine when canary nestlings first fledged.

Twenty nine canary nestlings were hand-reared and observed for fledging behavior from P7 - P18 at lights on, during midday (1100 - 1400), and at lights off. The onset of fledging was defined as either perching on the side of the nest at rest or during feeding (Figure 2.24A). Until that point, nestling spent all of their time inside the nest's bowl.

Results & Conclusions:

The results are striking and show that for the vast majority of canaries, the onset of fledging is P16. Of the 29 nestlings followed, 2 showed fledging postures at P15 (notably during the evening assessment), 1 fledged at P17 (evening assessment) and all 26 remaining nestlings fledged at P16 (Figure 2.23B).

What is perhaps most exciting for me is that this result ties together all of the previous findings. The emergence of a new locatable call type that is produced in contact-call-like settings makes perfect sense in the life-history of the nestling if this is when the animal will for the first time stand away from nest but

A



B

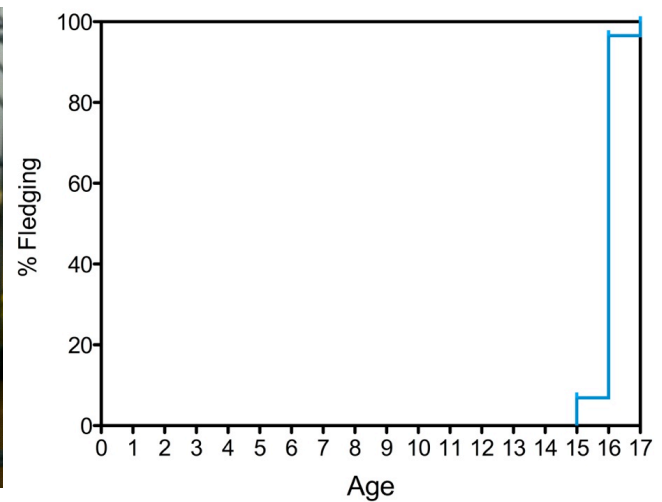


Figure 2.23: Fledging occurs largely at P16. A) Two canaries in the nest they were hand-raised in. One P16 fledgling is perched on the edge of the nest and another P15 nestling is sitting in the nest. **B)** Graph displaying the percent of birds that have fledged, as defined by age. Two birds fledged at P15 in the evening, 26 birds fledged at P16 and one at P17.

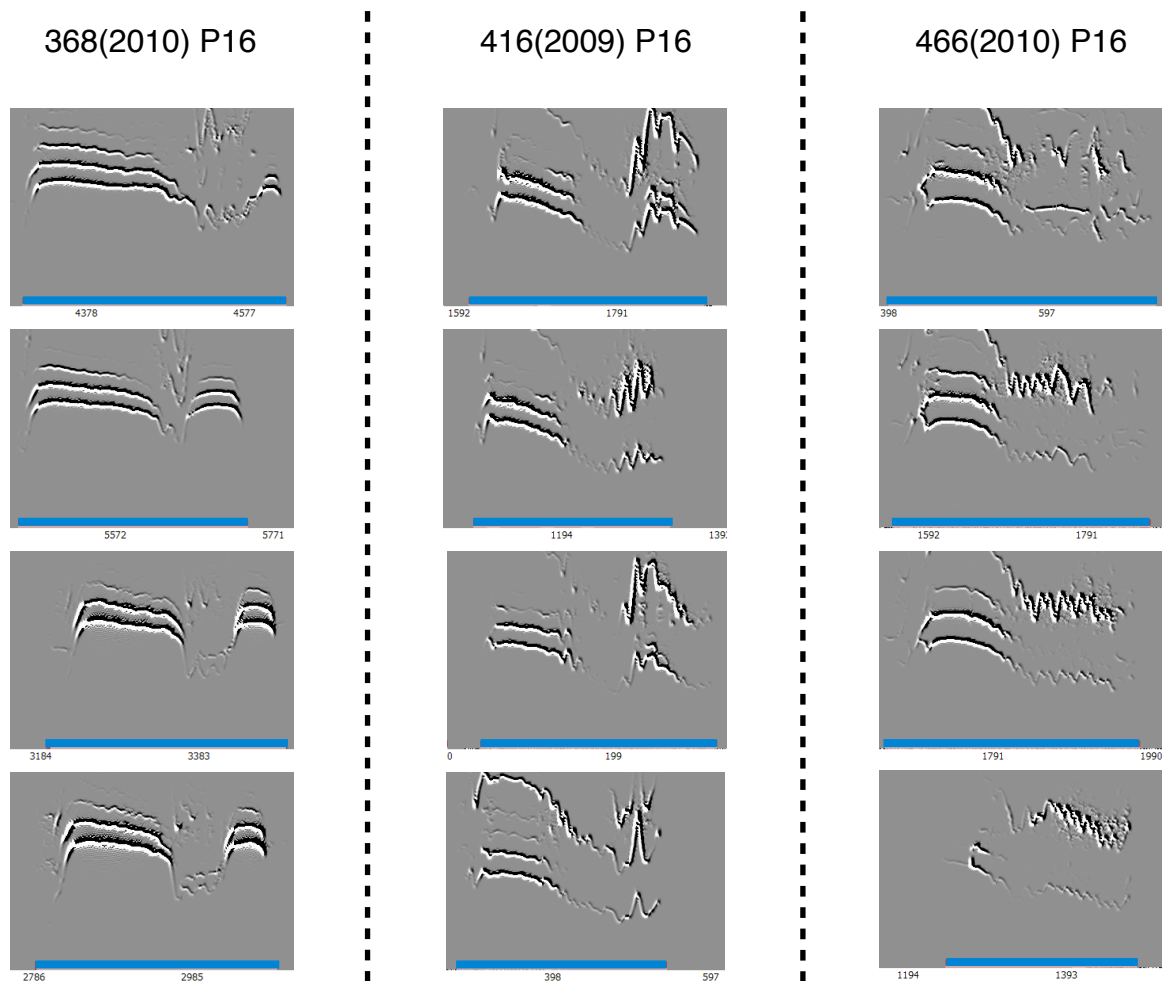


Figure 2.24: B calls. The first four B calls recorded from each of three P16 birds are displayed above in columns. Note the differences in calls within a bird and also between birds. Bird 368 (Left Column) has a highly modulated portion of the call in the middle of the call while the frequency undulations come at the end in birds 416 (Middle Column) and 466 (Right Column). All calls are scaled similarly. Numbers below sonograms represent milliseconds. Distance between numbers is ~200 ms.

still require feeding from parents. More exuberant visual and vocal begging displays, which have been noted to occur when birds are older (Kilner, 2001; Leech & Leonard, 1996; McCarty, 1996), may also be predicted if the bird must now compete with siblings for the attention of parents and scarce food resources across much greater physical space than the nest. The result of this greater competition once the nest is left poses a new conflict for young. If perching outside of the nest lowers the chance of being fed relative to those that are still inside the nest because of proximity to parents, nestlings are under evolutionary pressure to stay in the nest as long as the parents will permit, regardless of their physical capability to perch or not. Of course, the cost of leaving the nest must be balanced with the cost of staying in the nest too long, which is at higher predation risk as it is a fixed post of recurring activity that predators might more easily notice and investigate than freely moving -potentially flying- animals. One way to resolve this conflict might be to produce calls that successfully signal location and then, if needed, identity. Nests with louder and/or more persistent begging sounds suffer greater predation risk (Briskie et al., 1999; Dearborn, 1999; D.G. Haskell, 1994; Leech & Leonard, 1997). Thus, conspicuous and easily locatable begging calls may induce feeding by parents because they signal nutritional need that the parents are predisposed to feed (H. C. J. Godfray, 1991; Kilner & Johnstone, 1997; Smiseth & Moore, 2002) or because parents are under evolutionary pressure to quiet any individual that may reveal the nest location. Perhaps parents do not need locatable calls to spot their fledged offspring, but the young have evolved such calls in order to dare parents not to feed them.

Whatever the specific case, the current correlative data suggests that B calls may arise to serve new communicative needs that come with leaving the nest.

Experiment 10: Can B calls signal individuality?

Contact calls in other species of birds have been shown to carry individuality signals (Buhrman-Deever et al., 2008). For example, budgerigars (see Appendix 4) can discriminate over 30 individuals based on their contact calls (Ali, Farabaugh, & Dooling, 1993; Dooling, 1986). If B calls do serve to beckon parental feeding, a signature of identity may be necessary, especially as location after fledging no longer guarantees relatedness. To appreciate B call diversity between individuals, see Figure 2.24. To assess whether the structure of B calls alone could distinguish individual nestlings, every B call produced (30 - 39 calls per individual) by each of five P17 fledglings was collected and analyzed in SAP for call features. Averages for each bird for each feature were calculated and compared to one another using a One-way ANOVA and Tukey's Multiple Comparison Test.

Results & Conclusions:

Type B calls can significantly differ between birds. A One-way ANOVA revealed significant differences in **call duration** $F(1, 5) = 46.90, p < 0.0001$ (Figure 2.25A), **average pitch** $F(1, 5) = 35.57, p < 0.0001$ (Figure 2.25B), **average frequency** $F(1, 5) = 9.105, p < 0.0001$ (Figure 2.25C), **average**

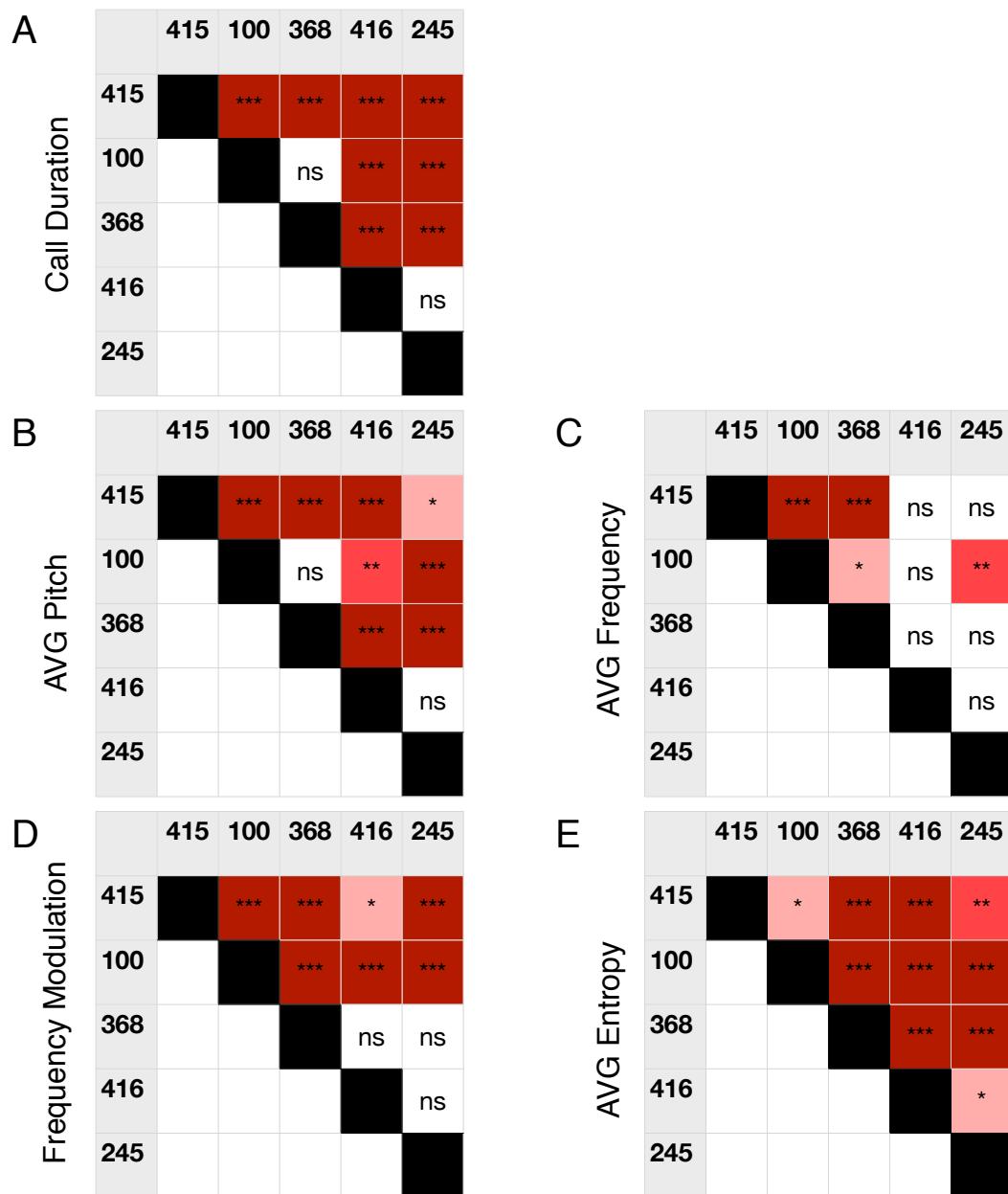
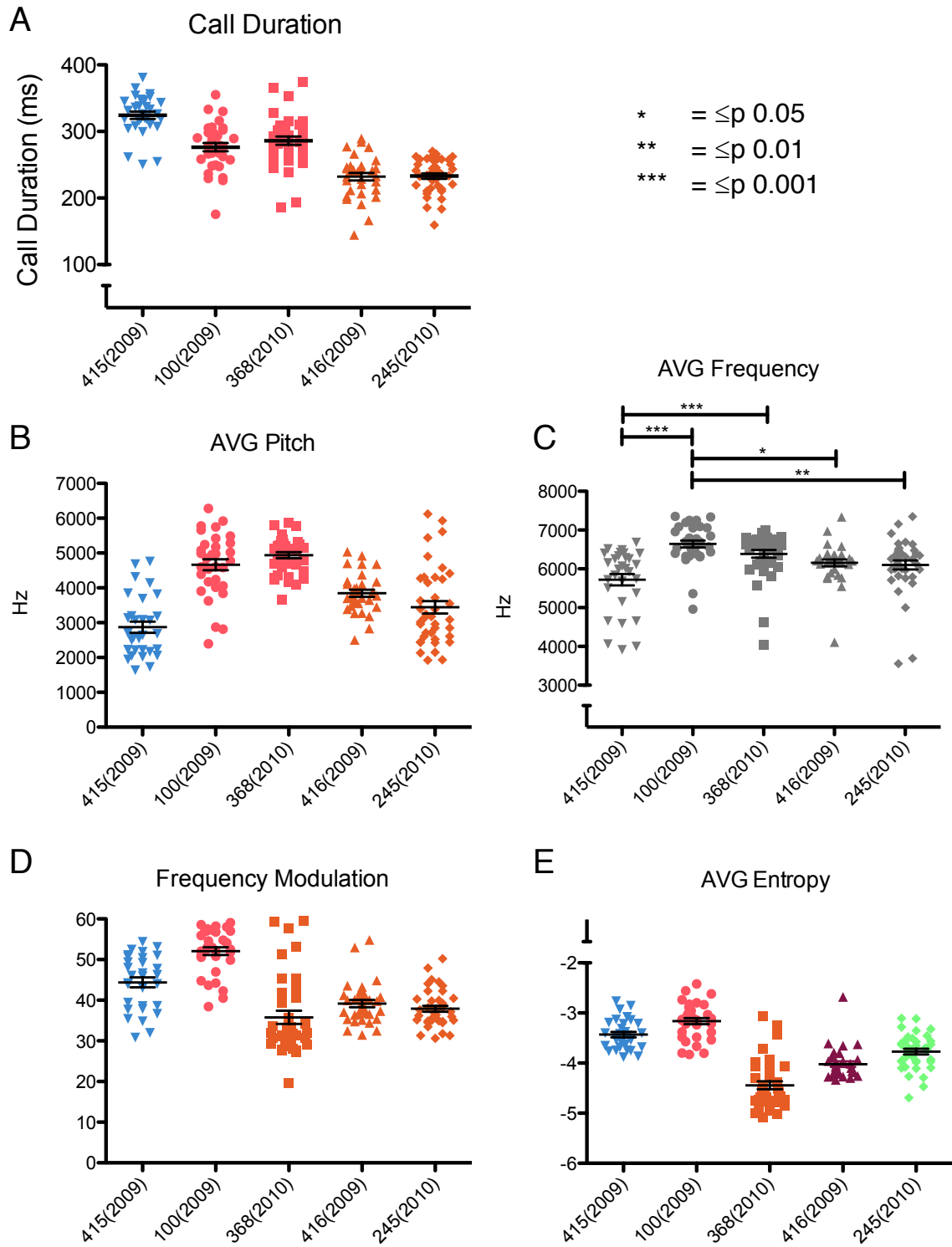


Figure 2.25: The characteristics of B calls significantly differ between individual birds. 30-39 B calls from each of five P17 individuals were collected, analyzed for call features using SAP and are displayed above. Averages \pm Standard Error shown. Birds are color coded where possible to reflect statistical differences whereby differently colored birds are statistically different ($p \leq 0.05$) from each other. Statistical comparisons between birds for all features are displayed below.

Figure 2.25



frequency modulation $F(1, 5) = 32.92$, $p < 0.0001$ (Figure 2.25D), and **average entropy** $F(1, 5) = 62.74$, $p = <0.0001$ (Figure 2.25E). Tukey's post-hoc comparisons revealed significant differences between individual birds.

The features of B calls, like A calls, can be used to statistically distinguish individuals. In fact, every feature assessed significantly differed between some birds and as little as one feature (average entropy) distinguishes all five tested individuals. Intriguingly, two of the birds were siblings 415(2009) and 416(2009) and their calls differed in every measure assessed except average frequency, suggesting that even genetically related nestlings can produce very distinctive calls. Of course, using these particular call characteristics to distinguish birds does not mean that canary parents use these specific call traits to distinguish their young, simply that vocal signatures are present -whether they are heard or not. Further experiments should be carried out to examine whether canary parents distinguish their young using B calls and which call characteristics are used to discriminate begging individuals.

Experiment 11: How do B calls arise?

Vocal learning in songbirds progress through what many thought were three stages of vocal learning culminating with adult song (Figure 1.6). First, birds produce randomly ordered and highly variable syllables, much like a child's babbling, in a stage called subsong. These syllables are then slowly modified

through a stage called plastic song, during which some syllables are modified to match a model and their repetition becomes gradually more stereotyped.

Eventually, these syllables become highly stereotyped and coalesce into a highly ordered structure, a process referred to as crystallization, whereby the resulting adult song will be kept for life in some species (zebra finches for example) or for a single breeding season in others (canaries and other seasonal breeders).

Throughout the process of song learning, vocalizations are modified, stereotyped, and organized to achieve greater complexity. In other words, in vocal learners, one stage of vocalization arises from the modification of vocalizations from the prior stage. While the three-stage model of vocal learning has largely remained unchallenged, newer work has suggested that perhaps food begging calls, the earliest vocalizations produced by birds, may begin the process that leads to song (Liu et al., 2009). For example, in a number of vocal learning species, food-begging calls are sexually dimorphic (M. E. Hauber & C.K. Ramsey, 2003; Liu et al., 2009; Saino et al., 2003; Saino et al., 2008), may utilize forebrain circuitry (Heaton & Brauth, 2000a, 2000b; Liu et al., 2009) and use auditory information (Heaton & Brauth, 1999; Liu et al., 2009). The proposal that food begging calls may be the start of vocal learning, while still not widely accepted, would represent a further example of early vocalizations leading to more complex ones. I described earlier that B calls appear suddenly in ontogeny (Figure 2.21) and I sought to investigate *how* B calls emerged, whether they had no previous precedent or whether they followed the wider pattern of being modified vocalizations.

To first visually examine the onset of B calls, I recorded seven canary nestlings while food begging from P15 until they either reached P21 or stopped vocalizing during food begging. Every recorded vocalization was visually examined and the appearance of the first and last B calls produced were noted for each bird.

Five of the seven nestlings produced B calls. However, all five birds produced their first B call at P16. Intriguingly, the B calls produced on the last day of food begging were structurally different from the earliest B calls produced (Figure 2.26), suggesting that the B call itself is modified over time. What was not expected however, was that the first B calls produced appeared to be constructed of A calls that had a highly frequency modulated component added (Figure 2.26). To test whether the first portion of B calls truly resembled A calls, I analyzed the beginning of B calls (Figure 2.26, between the yellow lines) and the A calls for call characteristics and then compared the two calls to each other within each bird. The first 10 A and B calls produced at P16 were analyzed so as not to bias data collection. The duration, average pitch, average frequency, average entropy and average frequency modulation were statistically compared using an independent-samples t-test with a Bonferroni adjustment made to the alpha level to protect against Type 1 errors. A new p level of 0.01 ($0.05 / 5$ birds) was set as the significance threshold.

Results & Conclusions:

A calls were significantly different in **call duration** than the beginning of **B** calls for only birds 167 and 292 (Figure 2.27).

A calls were *not* significantly different in **average call pitch** than the beginning of **B** calls for any bird analyzed (Figure 2.27).

A calls were *not* significantly different in **average call entropy** than the beginning of **B** calls for any bird analyzed (Figure 2.27).

A calls were significantly different in **average call frequency modulation** than the beginning of **B** calls only for birds 415 and 158 (Figure 2.27).

A calls were significantly different in **average call frequency** than the beginning of **B** calls for only bird 167 (Not pictured).

While there were some differences in some features, in two of five birds (158, 156), there were no differences in *any* analyzed call feature. Moreover, there were no differences found in any bird between **A** calls and the start of early **B** calls in entropy or frequency modulation, two features that differ significantly between **A** and **B** calls (Figure 2.18). In fact, the **A** and **B** calls are so distinct in call features that the large amount of similarities found between the P16 **A** call and the first portion of the early P16 **B** call are striking, leading us to conclude that the first **B** calls produced are structurally **A** calls with an attached **B** call modification. Thus, provocatively, the present data suggests that **B** calls may first arise as modifications of **A** calls.

Figure 2.26: Portions of early B calls visually resemble A calls. The A and B calls of five different P16 birds are displayed. The bird number is located on the top left of each row of sonograms. P16 was the first day that B calls, underlined in blue, appeared for all five individuals. Note that the a portion of the each bird's B call, highlighted between yellow vertical lines, visually resembles the A call, highlighted in red. The other portion of B calls is marked by higher frequency undulations (birds 415, 167), noisy sounds (158, 292), or strange and never again replicated long whistles and undulations (156). Within a few days, every bird produces highly undulated B calls, underlined in purple.

Figure 2.26

A and Early B Call

Final B Call

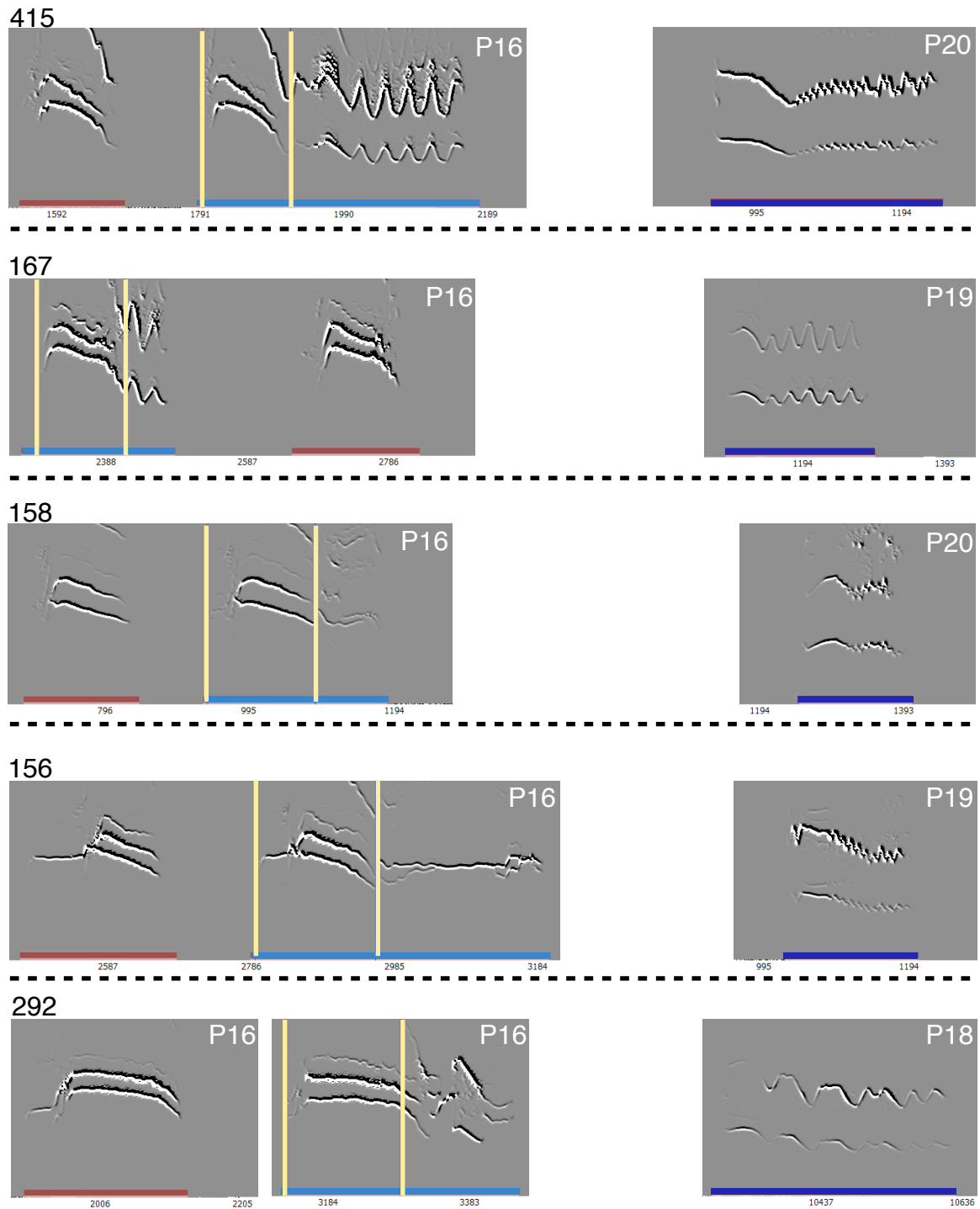
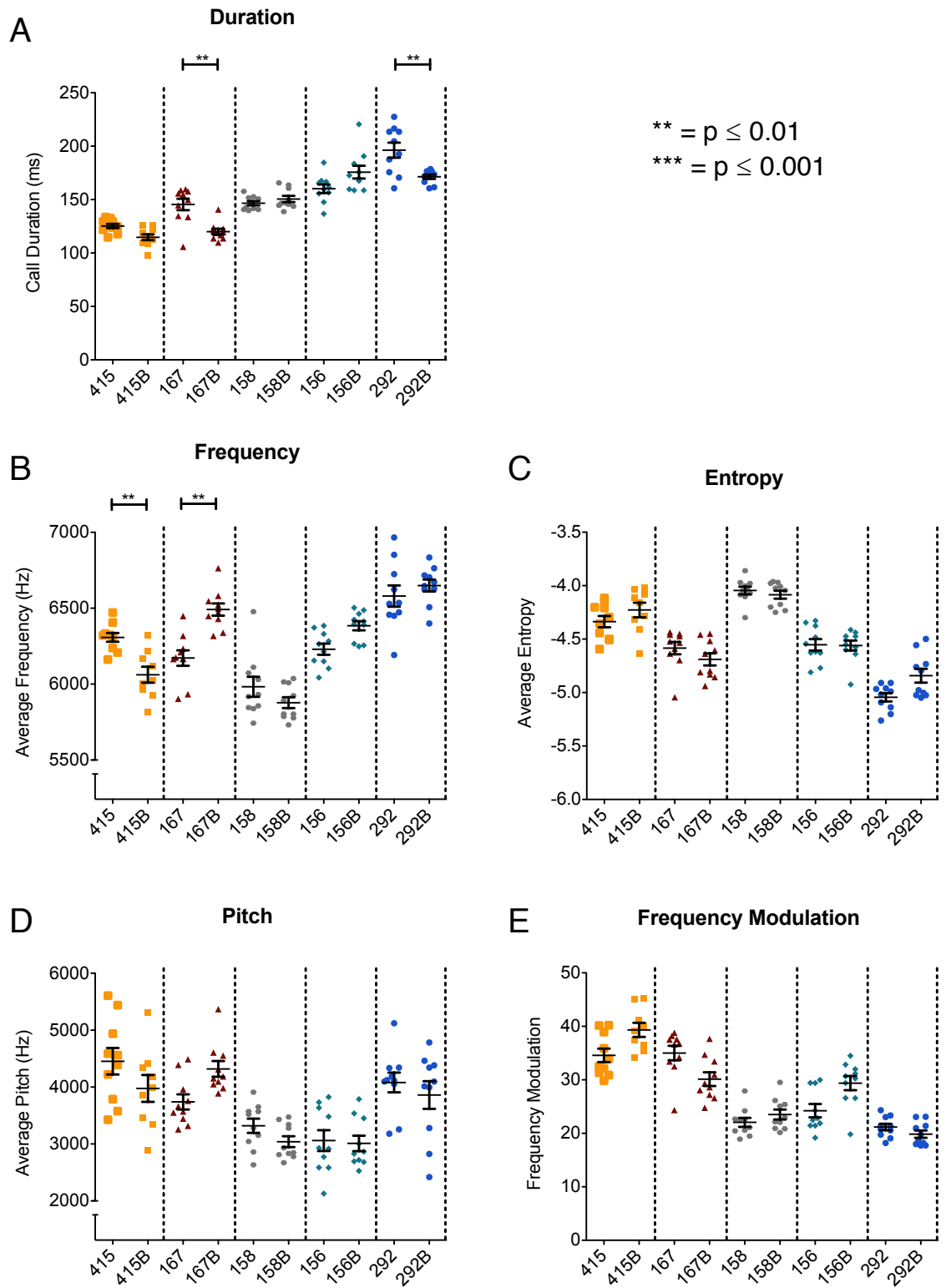


Figure 2.27: Portions of early B calls have similar call characteristics with A calls. Ten A calls for each of five P16 birds and ten visually-similar portions of B calls from those same individuals were analyzed for call characteristics using SAP. The results for call duration (**A**), average frequency (**B**), average entropy (**C**), average pitch (**D**) and average frequency modulation (**E**) for the begging call and contact call portion for each bird are displayed. Averages \pm Standard Error shown. Birds are separated by dotted lines and are marked by different colors. Within each column, the data on the left are the measurements from the A call and the data on the right are the measurements from the B call. The five birds analyzed are those pictured in Figure 2.26.

Figure 2.27



Experiment 12: Are A and B vocalizations produced similarly?

Type B calls are structurally distinct calls from all other food begging calls produced by a canary (Figures 2.1, 2.17, 2.19). We were struck by the structural complexity of these B calls and particularly the rapid frequency undulations which suggested to us that they were perhaps produced by syringeal modulation. The rapidity and spectral range of the frequency modulations in B calls were so unlike those found in A calls that we wondered if perhaps the production of these two calls differed. While developmental changes in the structure of begging calls has been noted before in other species (Anderson et al., 2010a; Anderson et al., 2010b; J. Clemmons & Howitz, 1990), no studies have to this date been undertaken to understand the peripheral mechanisms of food-begging call production.

There are three predominant ways that birds can control the frequency of vocal sounds produced. First, birds can adjust the tension of the medial and lateral labia while air flows between them (Figure 1.4) and this musculature-driven method allows for rapid and fine control of vocalizations (Goller & Larsen, 1997; Goller & Suthers, 1996a; Larsen & Goller, 1999; Suthers, Goller, & Pytte, 1999). This is the predominant method in which adult songbirds alter the frequency of their vocalizations (Suthers, 1990; Suthers, 1997; Suthers et al., 1994; Suthers, Goller, & Hartley, 1996; Suthers et al., 2004). Secondly, instead of

modulating the labia, birds could maintain the labia at a constant tension but change the rate of airflow over them by modulating the abduction of the sternum, forcing air to more slowly or rapidly escape out of the airsac system through the syrinx. This method would work to modulate frequency because at a single tension, changes in airflow through a slit in a vibrating membrane lead to corresponding changes in sound frequencies being produced. As a result, at an unchanging syringeal tension, modulating thoracic pressure alone would result in frequency changes in the vocalization produced. This method of frequency modulation has not been well characterized in songbirds but we would hypothesize that the necessity to modulate the large muscles used in expiration to achieve airflow changes might result in slower frequency modulation capabilities as well as less fine frequency control compared to modulating the significantly smaller syringeal labia. Thirdly, other, perhaps upper vocal tract (or post-syringeal) mechanisms may play a role in sound production (Nelson, Beckers, & Suthers, 2005; Riede & Suthers, 2009; Zollinger, Riede, & Suthers, 2008) but because syringeal use is the predominant method for frequency modulation, we began by testing the first two methods of sound production. Specifically, I hypothesized that type A calls could conceivably be produced by either syringeal or expiratory modulation -or both. However, B calls, might require participation of the syringeal musculature to achieve the rapid frequency modulations observed. To test this hypothesis and better determine the method of sound production in A and B calls, I collaborated with Dr. Roderick Suthers at Indiana University. I drove nestling canaries to Indiana University where we

measured thoracic pressure during food-begging call production. Animals were audio recorded during all measurements.

Pressure Cannulae:

The airsac pressure produced during breathing and vocalization is measured by implanting a flexible silastic cannula (Dow Corning Corp, Midland, MI, USA; i.d. 1.02 mm, o.d. 2.16 mm) into the cranial thoracic airsac. Nestlings were anesthetized using isoflurane and a small incision into the body cavity was made 1 cm from the caudal edge of the sternum and the prepared cannula inserted into the cranial thoracic airsac. The cannula was secured via sutures between the posterior two ribs and tissue adhesive applied close the incision further secured the cannula. The external end of cannula was attached to a miniature piezoresistive pressure transducer (Fujikura FPM-02PG, Marietta, GA, USA) mounted on a small backpack the birds carry via an elastic belt. In this manner, we were able to assess the relationship between expiratory/inspiratory pressure and the resulting vocalization recorded by the microphone in a freely moving canary fledgling.

Analysis

To test whether thoracic pressure could account for frequency of call production, I measured the thoracic pressure and call frequency at various points across A and B calls (Figure 2.28). Igor Pro 5.05 and additional software written for it by Brian Nelson (Indiana University) were used to visualize and measure

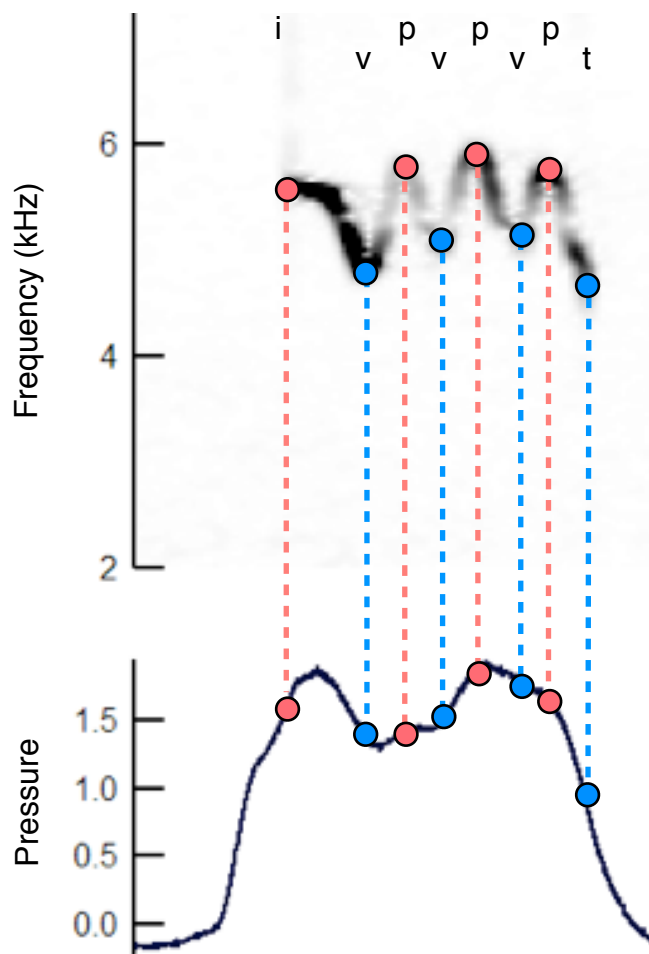


Figure 2.28: Measurements of thoracic pressure and frequency of calls produced. One B call and simultaneous thoracic pressure trace shown above. Call frequency and thoracic pressure were measured at the initiation (i) and termination (t) of the call, as well as all frequency peaks (p) and valleys (v) of begging and contact calls. 8 - 13 calls were measured in each of 3 animals. Colored dots above signify where along the call frequency and pressure measurements were taken. Red and blue are only used to help the eye match measurements between the two waves.

data collected. For each of 3 nestlings, 8 - 17 calls of each type were measured. The change between adjacent points of both frequency and pressure were calculated so see the relationship between changes in thoracic pressure and frequency. For example, we may ask, as the thoracic pressure rises during a call, does the frequency of the call also rise? These changes were then plotted and a Pearson correlation analysis conducted to see if there was a relationship between changes in thoracic pressure and changes in call frequency for both A and B calls in all three birds.

Results & Conclusions:

Pearson correlations revealed a strong, positive correlation between changes in thoracic pressure and call frequency in A calls in all three birds. Bird 123: $R^2 = 0.781$, $n = 52$, $p < 0.0001$. Bird 133: $R^2 = 0.9384$, $n = 24$, $p < 0.0001$. Bird 134: $R^2 = 0.8780$, $n = 18$, $p < 0.0001$ (Figure 2.29). Oppositely, within B calls, correlations between thoracic pressure changes and frequency changes were weak in bird 123 ($R^2 = 0.1782$, $n = 55$, $p = 0.0013$) and bird 133 ($R^2 = 0.1793$, $n = 66$, $p = 0.0004$) and not significant in bird 134 ($R^2 = 0.0044$, $n = 76$, $p = 0.5701$; Figure 2.29).

Changes in thoracic pressure account for an average of ~87% of changes in frequency in A calls but only an average of ~12% in B calls, as calculated from R^2 values. In other words, type A calls can be largely explained by thoracic pressure changes but B calls can not. The present data support a model where

frequency of **A** calls is predominantly a result of expiratory pressure with the syrinx behaving as a passive organ. Now, how are **B** calls produced?

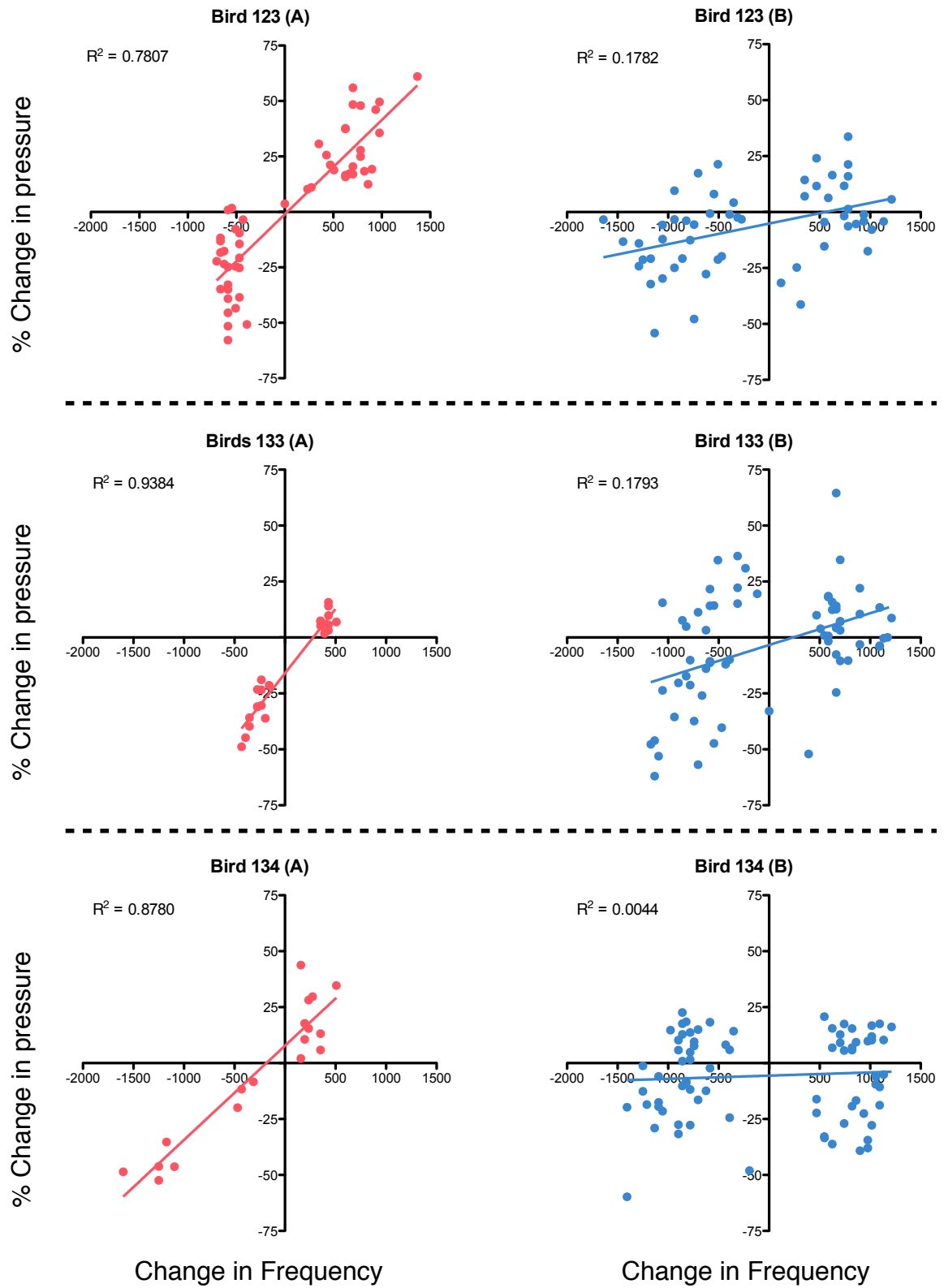
Unfortunately, the current data does not answer that, it only rules out modulations of expiratory pressure as a major contributor. While we do not yet have direct evidence, we hypothesize that the rapid frequency undulations characteristic of **B** calls might be under syringeal control. Indeed, the rapidity of the modulations, particularly in the final **B** call (Figure 2.26), support the likelihood of a very rapidly modulated muscle, in the case of songbirds, likely in the syrinx. My earlier findings that **B** calls may arise as modifications of **A** calls, may suggest that the early **B** call is constructed of an expiratorily-driven **A** call with the addition of a syringeal-driven frequency modulation. Later studies presented in this thesis utilizing unilateral denervations of the syrinx may further our understanding of the peripheral mechanisms of begging call production.

Final thoughts

This chapter describes the vocal ontogeny of begging in canaries. Herein I document the two major call types, their call characteristics, how they change over time, the potential role of each call type through behavioral experimentation and structural analysis of the calls. Interestingly, the **A** and **B** calls may have different communicative roles, with **A** calls serving a more traditional begging call function while **B** calls may additionally be used as contact-like calls. I have furthermore acquired evidence for call discrimination abilities in adult female

Figure 2.29: Changes in pressure correlate strongly with changes of frequency in A calls but not B calls. The correlation of percent change in thoracic pressure (Y-axis) and changes in call frequency (X-axis, Hz) for A and B calls in three birds are shown above. In all three birds there is a strong positive relationship between thoracic pressure and call frequency in A calls (See left column) but not B calls (See right column). Note, for example, how positive changes in pressure results in higher frequencies in A calls but not necessarily in B calls. R^2 values for each correlation shown.

Figure 2.29



canaries -considering the call variability within individuals, this ability is rather impressive. Collectively, these studies provide a foundational body of work for future studies of begging calls in canaries and other songbirds. In addition, it provides a basis for research on the origins of left vocal dominance in canaries.

Chapter 3: The emergence of lateralization in food-begging calls

I chose the songbird for my thesis work because of the well-described anatomy of the song system that made it a great model for brain laterality. For example, songbirds have no corpus callosum, a long list of lateralized behaviors (Cynx, Williams, & Nottebohm, 1992; Facchin, Burgess, Siddiqi, Granato, & Halpern, 2008; Floody & Arnold, 1997; Greenspon & Stein, 1983; Halle et al., 2003; Koboroff, Kaplan, & Rogers, 2008; Liedvogel et al., 2007; Lieshoff, Grosse-Ophoff, & Bischof, 2004; Phan & Vicario, 2010; Voss et al., 2007; Weir, Kenward, Chappell, & Kacelnik, 2004; Williams, Crane, Hale, Esposito, & Nottebohm, 1992; Wiltschko, Traudt, Gunturkun, Prior, & Wiltschko, 2002), accessibility to peripheral methods to assess unihemispheric function (Hartley & Suthers, 1990; Nottebohm, 1971; Nottebohm & Nottebohm, 1976; Wiltschko et al., 2002), and discrete neuroanatomy that underlies vocal behavior (Nottebohm, 2005; Nottebohm & Liu, 2010a; Nottebohm et al., 1976; Suthers & Margoliash, 2002). Early denervation work (Nottebohm et al., 1979; Nottebohm & Nottebohm, 1976) suggested that lateralization of vocal learning began earlier than subsong. Recent studies showed that the late begging call and the early subsong utilized similar central processes, thereby proposing that the begging call was a “harbinger of song” (Heaton & Brauth, 1999, 2000a, 2000b). Building on these observations, I unilaterally denervated young canaries to explore how early vocalizations may be lateralized.

Experiment 1: Are begging calls lateralized?

Study 1A: Unilateral denervations in P7 - P8 canaries

To determine if food begging call production is lateralized at any point in food-begging ontogeny, I began by first unilaterally denervating young (P7 - P8) canary nestlings. Hatchlings (P4 - P6) were removed from nests within our colony (total $n = 9$; See 'Subjects' in Appendix 1), hand-reared (See 'Nestling feeding' in Appendix 1), and recorded (See 'Recording' in Appendix 1) throughout the duration of the experiment. When birds had reached appropriate age, I performed either a sham surgery ($n = 3$), or a unilateral denervation of the left ($n = 3$) or right ($n = 3$) tracheosyringeal (Ts) nerve.

Begging call recordings of pre- and post-denervation.

The following recording protocol was used for all denervation surgeries in this thesis unless otherwise noted. On the day of denervation surgery, the food-begging calls of birds were individually audio recorded throughout the day, including the last feeding before the lights were turned off. Two hours after the last feeding, birds were removed from the nest, denervated (see below), and returned to their nests before lights on. Throughout the following day, beginning with the first feeding at lights on, the food begging calls of every bird were re-recorded.

Denervation surgery

The following procedure was followed for all denervations presented herein unless otherwise noted. Two hours after the last feeding, individual birds were removed from their home nest, weighed and given anesthesia. Unless otherwise noted, all birds received 1:5 Nembutal (See '1:5 Nembutal' in Appendix 3) injections into the breast muscle at a dose of 5.7 μ l per gram of body weight. The post-operative anesthetic effects of Nembutal (impaired balance, muscle weakness, grogginess) generally lasted 3 ± 1.5 hours which can be a substantial amount of time for a nestling to be without food, especially if the animal must be food deprived for ~2 hours before anesthesia onset to prevent regurgitation of food during surgery. Thus, as I did not want nestlings to undergo up to 6.5 hours without food, I performed surgeries, unless otherwise noted, at night. Once the anesthetized bird did not react to a toe-pinch, the feathers around the neck on the side that was to be denervated were removed, exposing the skin. A small, (~10 mm), incision was then made halfway down the neck overlying the trachea. The trachea was exposed, the tracheosyringeal nerve (Figure 1.5) freed from the surrounding tissue and a ~2 mm stretch of the nerve was removed (Video 3). Finally, the neck incision was closed using tissue adhesive and the bird placed under a heat lamp to aid post-operative recovery. Sham surgery animals experienced the same procedures outlined above but did not have the nerve removed.

Results: P7 - P8

The begging calls early in development (P7 - P8) are typically quiet, relatively unmodulated and often appear as whistles, sometimes of one voice (Figures 2.1; 3.1A, C pre-denervation) or of two voices (Figure 3.1B pre-denervation). Sham surgeries had no visible effects on these early begging calls (Figure 3.1A). Similarly, birds unilaterally denervated during early development had few changes in the structure of their food begging calls regardless of side of denervation. Indeed, begging calls produced after left or right denervation still resembled typical food begging calls at this age (Figures 3.1B, C).

The persistence of two voices in the early begging call after unilateral denervation (Figure 3.1B) suggests that the begging calls of P7 - P8 nestling

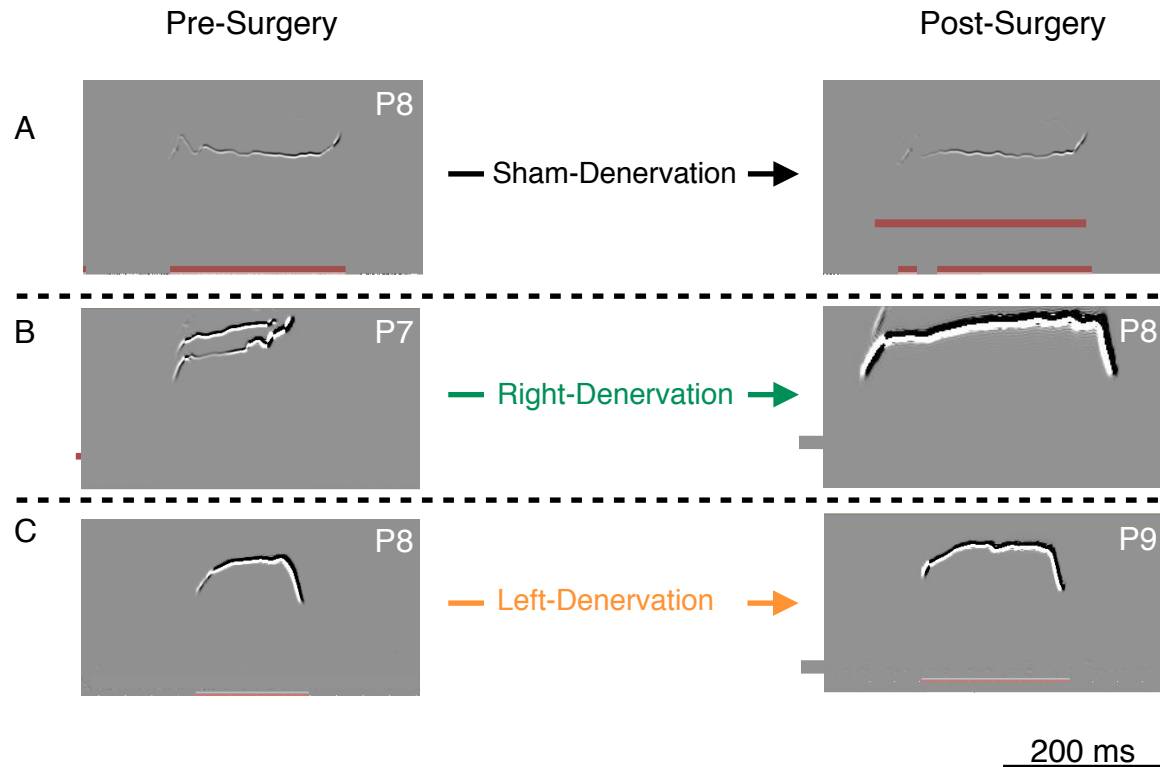


Figure 3.1: Unilateral denervations do not appreciably effect the structure of the earliest begging calls. The presurgery and post surgery begging calls for birds receiving sham (**A**), right (**B**), or left (**C**) denervations are shown above. Note that the general structure of the begging calls remain relatively unchanged. Birds in panels **A** and **C** produce a single-voiced call, while the bird in panel **B** produces a call with two voices. Note that the two voices remain after a right denervation. All pre-surgery sonograms are taken from the last feeding before denervation. The post-surgery sonograms are taken from the first feeding the following day.

canaries do not require the involvement of the asymmetrically innervated syringeal muscles. Indeed, the structure of early-produced begging calls is largely explained by changes in expiratory thoracic pressure (Figure 2.29). The present results further support a syringeal-muscle independent mechanism for the production of early begging calls.

Study 1B: Bilateral denervations in P8 canaries

In order to directly test whether the syringeal muscles actively contribute to the spectral qualities of the earliest begging calls, I performed bilateral denervations in four P7 nestlings, thereby inactivating the entire syringeal musculature. Nestlings were collected at P6 from nests in our breeding colony and hand reared throughout the duration of experiments. Bilateral denervations in adult songbirds (Nottebohm, 1971) and in older nestlings (personal observation) cause difficulty in breathing and in some cases, asphyxiation. Young (P7 - P8) nestlings, on the other hand, appeared behaviorally unchanged following surgery and gave no signs of discomfort.

Results and Discussion

In accordance with the study previously presented, bilateral denervations did not affect the begging calls of very young (P8) canaries (Figure 3.2). The four birds studied did not have muscular control of their syrinx and still produced vocalizations that closely approximated those made before surgery. In fact, even two voiced calls (Figure 3.2C, D) remained after bilateral denervation, supporting

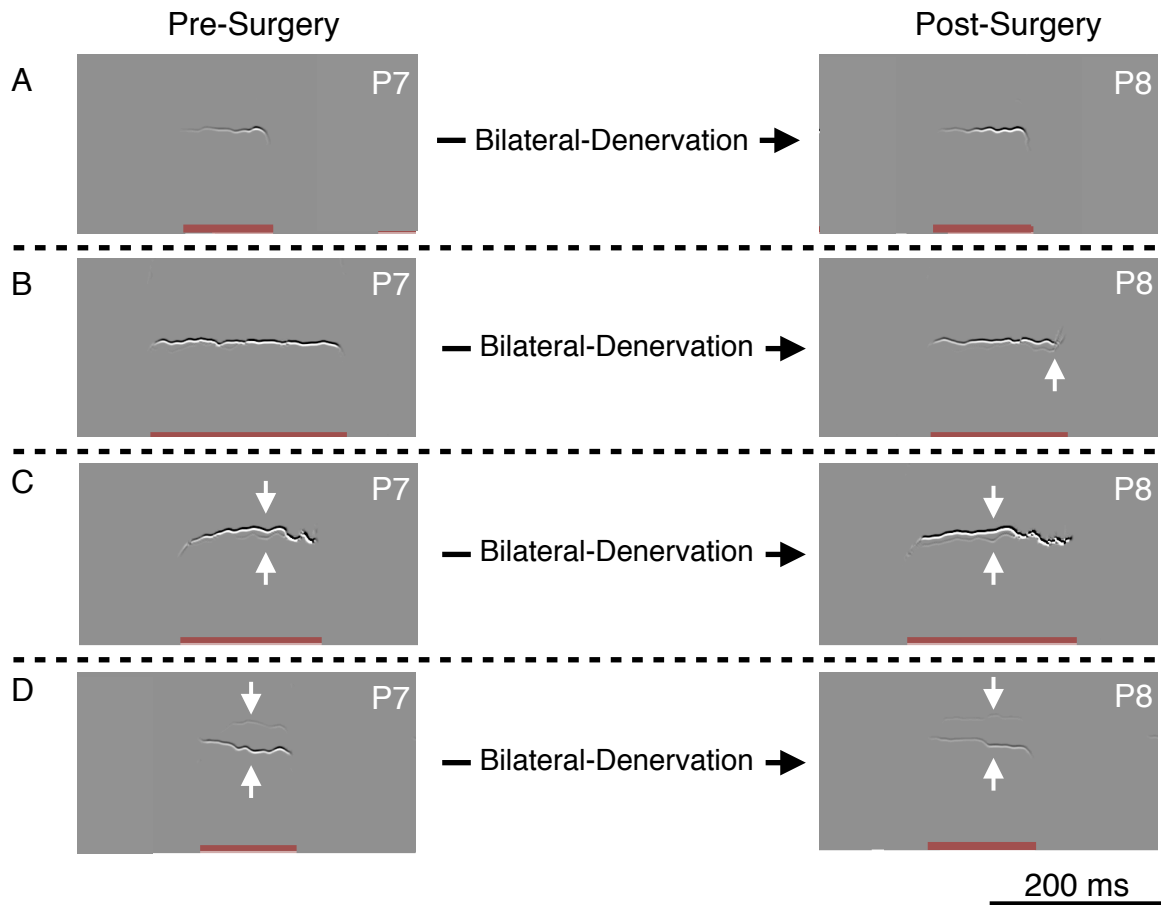


Figure 3.2: Bilateral denervations in young canaries have no identifiable effects on begging calls. The begging calls before and following bilateral denervations in four P7 - P8 canary nestlings. Single-voiced begging calls (**A**, **B**) appear unaffected. Begging calls that contain two voices before surgery (**C**, **D**; **arrows**) maintain them even with a bilaterally inactivated syrinx. Notably, one individual (**B**) appears to have gained a second voice following bilateral denervation (arrow, faint trace). All pre-surgery sonograms are taken from the last feeding before lights off. The post-surgery sonograms are taken from the first feeding the following day.

the interpretation that the very earliest calls, those that appear as relatively unmodulated whistles and which precede **type A** calls, are produced without the participation of syringeal muscles.

Study 1C: Are begging calls lateralized in almost independent nestlings?

In chapter 2, I showed that the structure of begging calls changes across development (Figure 2.1), and that new calls with different mechanical requirements appear after day 16 (Figure 2.21, Figure 2.29). To probe the neuronal contribution to these later calls, I denervated canary fledglings at different developmental ages.

Unilateral denervations in P19 - P20 canaries

I tested whether nearly independent (P19 - P20) fledglings produced begging calls in an asymmetric fashion by unilateral denervation of the tracheosyringeal nerve. Nine P6 - P9 nestlings were collected from our colony's breeding cages and were hand-reared until the termination of the experiment. Birds were then unilaterally denervated (left or right) or received sham surgeries (n = 3 per group) at P19 or P20.

Results

Sham surgeries had no meaningful effects on begging calls at these ages (Figure 3.3). Right denervations had minimal, but noticeable effects on the structure of begging calls (Figure 3.3B). In fact, what we see in the pre-surgery

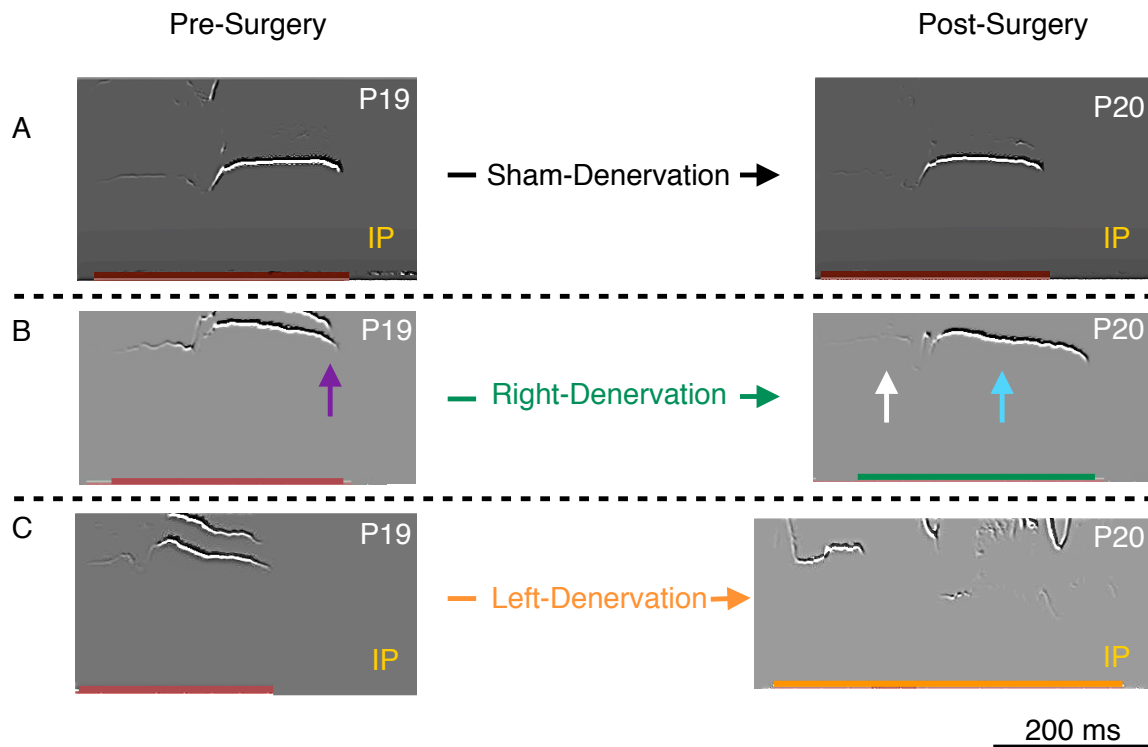


Figure 3.3: Left denervations late in begging ontogeny cause large changes to the begging call. All pre-surgery sonograms are taken at P19 from the last feeding before night. The post-surgery sonograms are taken from the first feeding the following day (P20). **B)** The purple arrow indicates the defining feature of a two-voiced call. Note also that the post-denervation call has only one voice, likely resulting from the silencing of the right sound source via denervation, but that the 2 element structure of A calls remains (white and light blue arrow). The sonogram images at P19 and P20 were processed (IP) to better visualize the quiet portions of the vocalizations. Please refer to the ‘Image Processing’ section of the thesis for more information.

sonograms in Figure 3.3B is a type A begging call with two voices (purple arrow: note how the top trace decreases in frequency faster and terminates earlier than the bottom trace), meaning that the bird is likely producing these separate sounds with two independent sound sources, presumably the left and right halves of the syrinx. Post-right denervation, one of the two voices disappears (presumably the right). However the structure of the call remains intact, with both A call elements still present (white and light blue arrows). Left denervations had dramatically different results: they became disorganized, lacked stereotyped structure and were produced at much lower amplitude (Figure 3.3C). In the example shown (Figure 3.3C), note that the post-surgery call, underlined in orange, does not at all approximate the pre-surgery call in structure.

An important note:

A difference between left and right denervations that can not be appreciated by studying sonograms is that left denervations sometimes resulted in markedly quieter vocalizations. This difference in call amplitude can not be fairly analyzed because in our recording protocol, the microphone distance to the beak varied between birds, before and after surgeries, etc, in order to adequately record the calls. Animals that begged quietly, because of being young or because of some surgical manipulation, were recorded with the microphone closer to the beak while louder animals were recorded with greater microphone-beak distances, meaning that measurements of amplitude are not reliable. I have included videos in the compact disc that accompanies this thesis of denervated

birds begging in order to better represent differences such as this that can not be adequately appreciated in sonograms and, whenever possible, to present more data of a raw nature for those that might be interested.

Experiment 1 Conclusions

There are two conclusions from the current set of experiments. First, the earliest begging calls are minimally altered by either left or right denervation. For example, two-voiced begging calls at P7 (Figure 3.1B presurgery) remained at P8 following unilateral denervation (Figure 3.1B postsurgery). Even more convincingly, *bilateral* denervations of P8 nestlings also left the earliest begging calls intact (Figure 3.2). It follows that if syringeal innervation is unnecessary in the early begging call, the syringeal muscles perform little or no role in the structure of these early calls.

The current denervation data suggest that the earliest begging calls (P7 - P9) are produced without the aid of syringeal musculature. Later in begging ontogeny, the fact that two voiced **A** calls lose one voice following right denervation (Figure 3.3B), suggests that the syringeal muscles play some role in these later begging vocalizations. However, the work I performed with Rod Suthers showed that the structure of **A** calls could be largely explained by changes in expiratory pressure and thus may not require active modulation of the medial and lateral labia by the syringeal muscles during the call (Figure 2.29). Our data suggests a model of begging calls developing mechanistic complexity

across ontogeny, with no requirement for syringeal musculature in the earliest produced vocalizations, and some syringeal contribution in the later produced **A** calls. If so, it is interesting to consider that across vocal ontogeny, from begging calls to adult song, not only does the complexity of vocalizations change from simple unmodulated calls to highly stereotyped and organized songs, but that the underlying mechanics may change as early as begging calls.

Second, and most interestingly, the two sides of the syrinx asymmetrically contribute to begging calls in late development. If unilateral denervation during this stage of development affected begging calls as later it affects song, then left denervations would have a greater effect on the call than right denervations. Moreover, right denervations, as in the case of adult song, affect the higher frequency voice (Figure 3.3B; Nottebohm, 1971; Nottebohm & Nottebohm, 1976).

Experiment 2: When and how do the asymmetric effects of denervation arise?

I next assayed the intermediate period between early (P9) and late (P20) begging calls for the onset of denervation effects.

Study 2A: When do left denervations wreck havoc?

To identify the ontogenesis of lateralized begging calls, I unilaterally denervated birds of all intermediate ages (P10 - P19) and subsequently

compared the pre-operative and post-operative begging calls and studied the resulting sonograms for asymmetric effects of denervation. A minimum of two birds per age (P10 - P19) per side (L or R) were used (total n = 43) as a first pass to better narrow down the range of when asymmetric denervation effects arise. Recordings and denervations were carried out as in experiment 1.

Preliminary results

Right denervations in intermediate ages never caused large disorganizations to the begging call, and had mild if any effects. Left denervation effects did not arise gradually, but appeared suddenly at P16 (denervated the night before at P15). Birds of older ages (P17 - P19) showed similar left-denervation effects (data not shown).

The sudden appearance of noisy, scraggly calls at P16 after left tracheosyringeal denervation warranted replication and so 6 - 13 birds per denervation group (L, R, Sham), per age (P15 and P16) were collected (total n = 61) and treated as in experiment 1. Male and female canaries are not externally sexually dimorphic at this age and so to equally represent each sex in each group, feathers were collected from each individual 2 - 4 days before denervation surgery, DNA extracted, purified (See 'DNA purification' in Appendix 3) and a PCR reaction run to sex the birds (See 'Sexing PCR' in Appendix 3 for details). This information allowed me to include similar numbers of males and females in each group.

Results and conclusions

The asymmetric effects of unilateral denervation appear suddenly at P16. Left denervations at P16 cause a significant change in call structure in all birds examined in this experiment ($n = 13$; Figure 3.4). Whether begging calls have a typical (Figure 3.4A) or atypical (Figure 3.4F) morphology, left denervations cause large disturbances in their structure (Videos 4, 5). Moreover, the effects appear to be broad and cause changes in all calls produced during food begging (Figure 3.5). Specifically, inspecting an entire begging bout, it is difficult to determine which calls might be **A** or **B** calls, suggesting that both are affected after left denervation. In two birds (Figure 3.5D, E), at least two call types can be readily distinguished. For example, in bird D, the last three calls shown (arrows) appear structurally reminiscent of each other, with the calls highlighted by **blue** arrows differing from the call highlighted in **red** only in the termination of the call — a highly modulated component suggestive of a **B** call. As early **B** calls may structurally first appear as modifications of **A** calls (Figure 2.26), the call highlighted by a red arrow may be an **A** call. Whether or not that is the case, both calls are affected. Still, if call types may be able to distinguished in some birds following left denervation, the calls for the majority of birds are sufficiently altered as to be difficult to assign call type based on sonographic data (3.5A, B, C, E, F, G). Lastly, **A** and **B** food begging calls vary significantly between individuals (Figures 2.2, 2.24) and the effects of left denervation at P16 similarly results in a wide diversity of call structures. Some fledglings produce noisy disjointed calls with significantly altered structure (3.4E) and others produce atypical whistles

flanked by noisy sounds (Figure 3.5F). Importantly to note, there were no sex differences in the effects of denervation. All P16, both left denervated males and left-denervated females had aberrant call structures (See males A, F, G, and females B, C, D, E in figures 3.4).

In contrast to the effects of left denervations at P16, individuals that are left denervated just one day earlier, at P15, do not show dramatic changes to the structure of begging calls (Figure 3.6). For their part, right denervations at P16 do not cause dramatic effects either (Figure 3.7, Videos 6, 7). In fact, the begging calls are so robust following right denervations that even very atypical begging calls with complex frequency modulations remain almost unchanged following surgery (Figure 3.7G). However, right denervations do have subtle effects. The first element in **A** calls is often shortened in duration (Figure 3.7B, C, D, E) and in some cases is replaced by high frequency sounds (Figure 3.7C, E). P15 left denervations cause similar effects on the post-operative call, with the first element of the **A** call being shortened in duration (Figure 3.6B, C, E, F, G).

Study 2B: Quantification of the effects of denervation at P15 and P16

The food begging calls of canaries are widely divergent between individuals (Figures 2.2, 2.5). Thus, as calls can vary widely between nestlings, with some producing calls with two voices (Figure 3.5B) and others with one voice (Figure 3.5G), some with little modulation (Figure 3.4D) and others with

Figure 3.4: Left denervations begin to cause large disorganizations in the structure of food-begging calls at P16. Each row (**A - G**) represents one bird before and after left denervation. The presurgery call structure after left denervation is largely unrecognizable. Both highly modulated (**F**) and relatively unmodulated (**D, G**) begging calls are severely affected. Yellow bars highlight the appearance of noisy runs in the post-surgery begging calls. These noisy portions can appear at the onset (**B, C, E, F**), termination (**B, C, D, E, F**) or middle (**A, C, G**) of the left enervated call. All pre-surgery sonograms are taken at P15 from the last feeding of the night. The post-surgery sonograms are taken from the first feeding the following day (P16).

Figure 3.4

200 ms

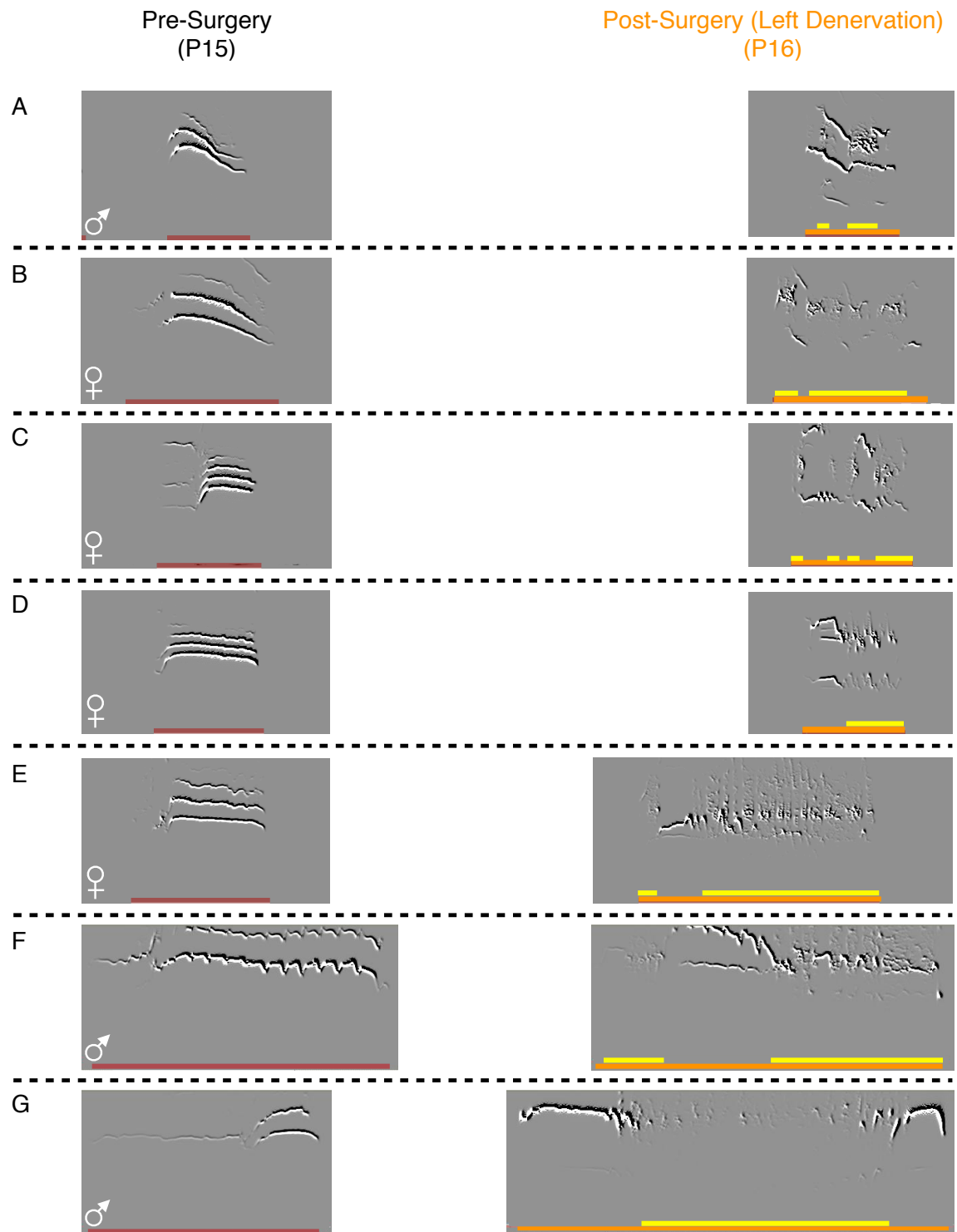


Figure 3.5: The effects of left P16 denervations across a begging bout.

Each row (**A - G**) represents one bird and the vocalizations produced during one begging bout in the first feeding session post-P16 left denervation. The first five vocalizations in the begging bout are shown, except for bird **F**, in which only the first three calls are displayed because each call's duration is so long that five calls could not fit on the page. All calls appear in the order they were produced and all continuous images are of continuous recordings. Wherever the five vocalizations would not fit on the page in a continuous image, the image was spliced (**A, C, F, G**). After left denervation, **A** and **B** calls are difficult to distinguish based on sonogram inspection in all birds. In two birds (**D, E**), we may perhaps be able to distinguish at least two call types post denervation. In bird **D**, the calls highlighted by a blue arrow appear to be derivatives of the call highlighted by the red arrow, with a similar structure at the beginning and the addition of a small highly modulated component reminiscent of a **B** call at the end. The first two calls that began the bout appear relatively distinct from the remaining three. In bird **E**, a short and long duration call can be easily distinguished, however the long duration call does not structurally resemble either **A** or **B** calls. Broadly speaking, the effects of left denervations appear to affect all calls produced throughout a begging bout.

Figure 3.5

200 ms

Post-Surgery (Left Denervation)
(P16)

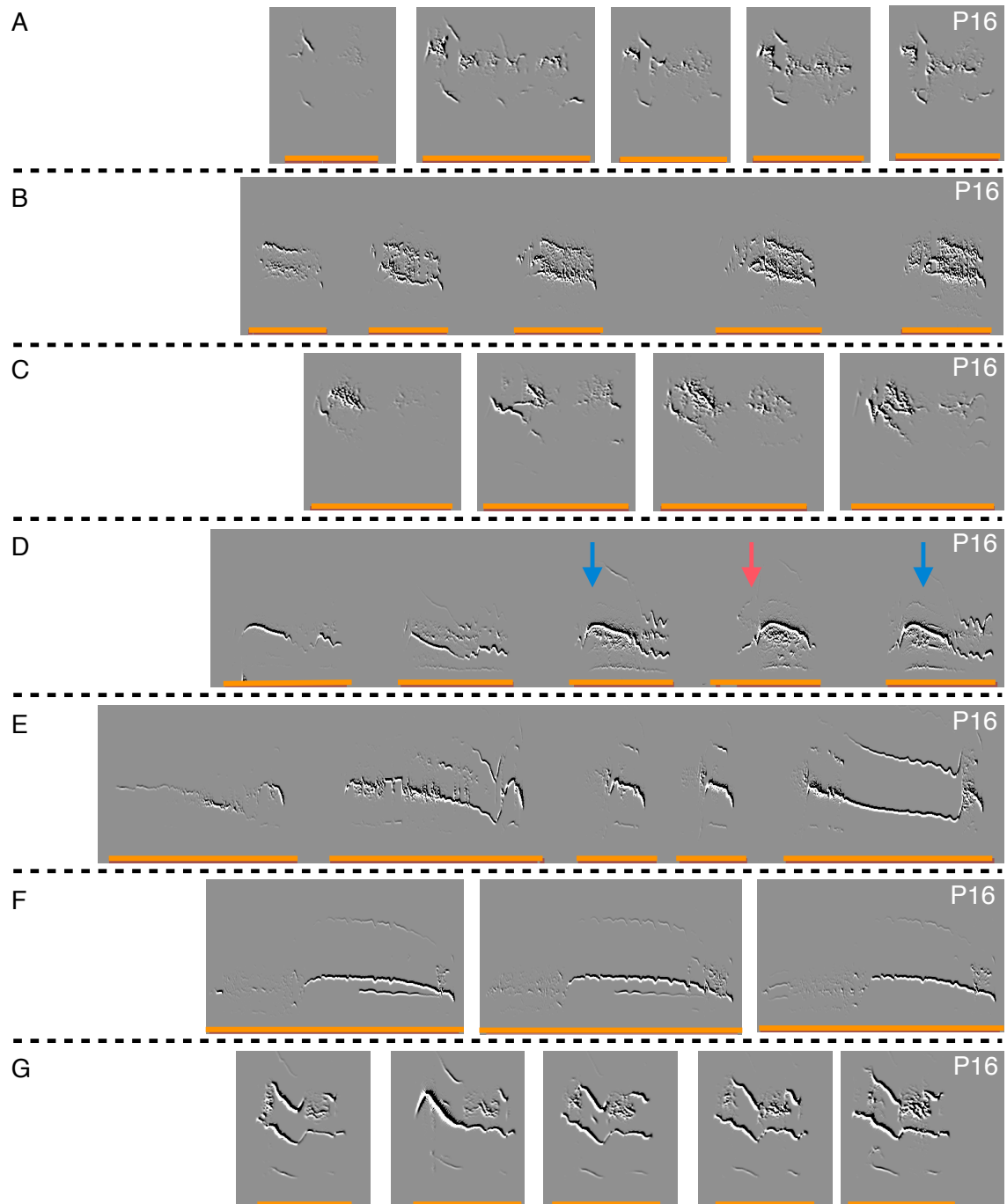


Figure 3.6: Left denervations have minimal effects on call structure at P15. Each row (**A - G**) represents one bird before and after left denervation. Unlike left denervations at P16, unilaterally removing the left nerve at P15 only partially disrupts the structure of begging calls. In fact, left denervations at this age had similar effects on calls as right denervations at P16 (Figure 3.4) and P15 (not shown). The call often becomes simplified and the first element of **A** calls is sometimes shortened (**C, E, F, G**) or is no longer present (**B**). All pre-surgery sonograms are taken at P14 from the last feeding before night. The post-surgery sonograms are taken from the first feeding the following day (P15).

Figure 3.6

200 ms

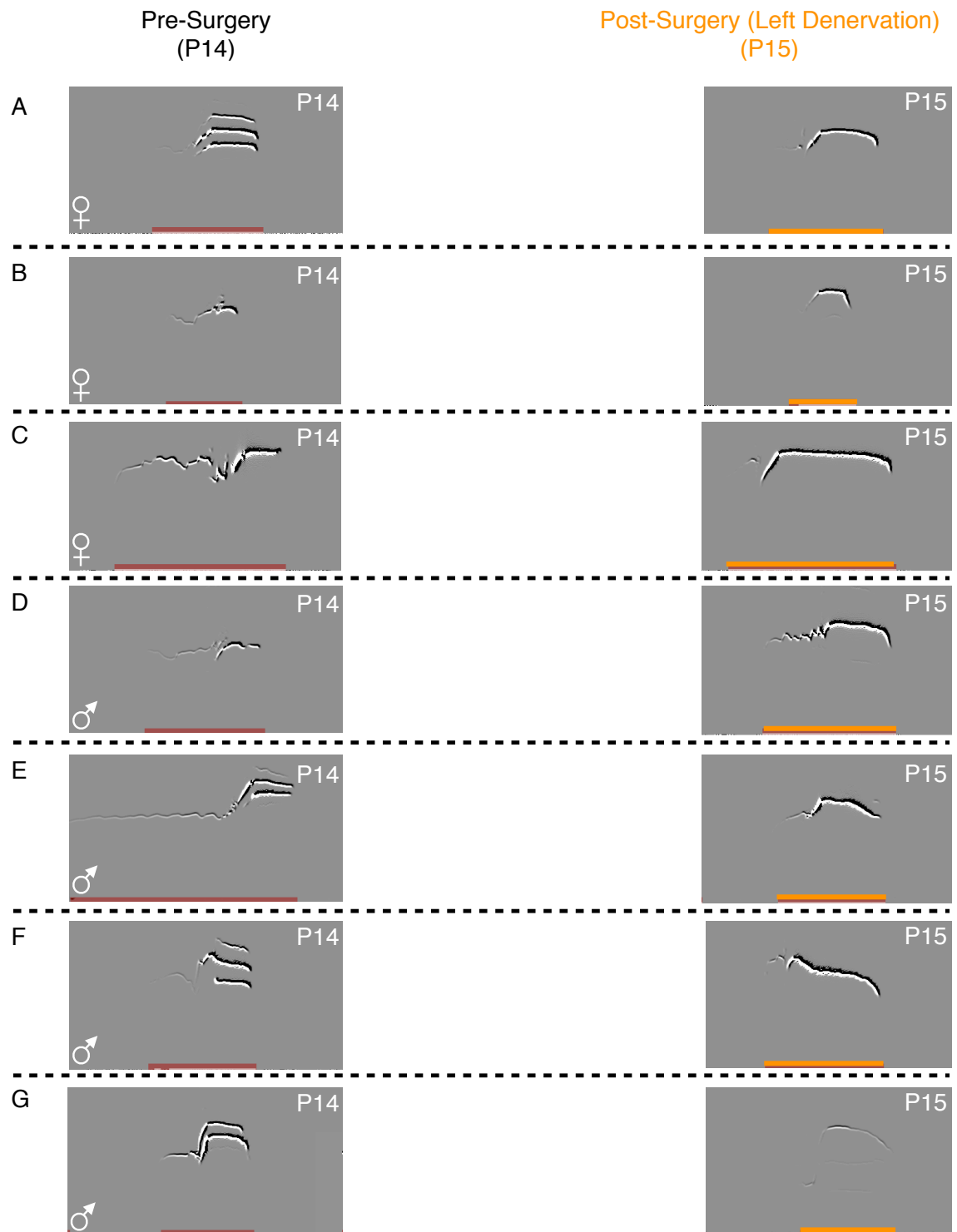


Figure 3.7: Right denervations at P16 cause minor disorganizations in the structure of the food-begging call. Each row represents one bird before and after right denervation. Note that though the call remains nearly indistinguishably intact post-denervation in one of the birds (**A**), others either lose quiet or noisy components (**F**, **G**: horizontal arrows), or have the quiet, first component of their **A** calls affected (**B**, **C**, **D**, **E**: vertical arrows). In all instances presented, the first element of **A** calls is noticeably shorter in duration and in some instances is partially replaced by high frequency sounds (**C**, **E**). However, unlike left-denervations, no noisy components appear nor aberrantly long or unstructured post-surgery begging calls. Thus, while there are minor affects to the calls in many right-denervated birds, the structure of the call remains largely intact. Indeed, even strange and highly structured pre-denervation food-begging calls like that seen in **G** are maintained post-surgery. All pre-surgery sonograms are taken at P15 from the last feeding of the night. The post-surgery sonograms are taken from the first feeding the following day (P16).

200 ms



much more modulation (Figure 3.4F), it is no surprise that the calls resulting from denervation also appear different between individuals (Figures 3.4, 3.7). That this diversity of call structures are all affected by left and not right denervations starting at P16 strengthens the results. However, it also makes visually interpreting how pre-surgery calls have changed after denervation more difficult. I therefore next quantified the effects of denervation on call characteristics at P16 and P15.

P16 Denervations:

Recordings from birds that had been denervated in experiment 2A were used in this analysis. All calls produced at P15 were used for pre-denervation call analyses and all begging calls produced at P16 were used for post-denervation analyses. Note that the denervation surgery occurred the night between P15 and P16. A minimum of 100 calls recorded for pre- and post-surgery were necessary for inclusion in the analysis. I manually curated every recording file of every bird and deleted any file that did not contain vocalizations and, from files that did, clipped out all unrelated noises (wing flapping, experimenter speaking, etc) to ensure that only calls were analyzed. Batch analysis settings in SAP were determined for each bird individually. For more details, please see 'Recording preparation' in appendix 1. Averages for call features were calculated for each bird before and after surgery. A paired-samples T-test was used to assess post-denervation effects for each call feature presented below. A Bonferroni

correction was used to account for alpha inflation and a new p value of 0.016 (0.05 / 3) was used to assess statistical significance.

Results:

A broad set of entropy and frequency modulation measures significantly increased in left-denervated ($n = 12$) birds, but not in right- ($n = 12$) or sham-denervated ($n = 9$) individuals. Table 3.8 summarizes all of the results found in detail below. For more details of call characteristics measured, see appendix 2.

Sham denervations:

Average call duration does not change following surgery. Presurgery ($M = 185.1$ ms, $SD = 45.98$ ms) and postsurgery ($M = 196.9$ ms, $SD = 70.30$ ms) calls are statistically similar in duration, $t(9) = 0.7307$, $p = 0.4858$.

Average call frequency does not change following surgery. Presurgery ($M = 6503$ Hz, $SD = 619.1$ Hz) and postsurgery ($M = 6424$ Hz, $SD = 625.9$ Hz) calls are statistically similar in frequency, $t(9) = 1.076$, $p = 0.3135$.

Average call entropy does not change following surgery. Presurgery ($M = -4.973$, $SD = 0.9833$) and postsurgery ($M = -4.971$, $SD = 1.004$) calls are statistically similar, $t(9) = 0.06368$, $p = 0.9508$ (Figure 3.9A).

Table 3.8

Call Characteristic	Left Denervation	Sham	Right Denervation
Average duration	0.5111	0.4858	0.2809
Average frequency	0.2899	0.3135	0.1081
Average entropy	0.0100 ↑	0.9508	0.1014
Peak entropy	0.0002 ↑	0.2602	0.0227
Standard deviation of entropy	0.0160 ↑	0.9158	0.2024
Average frequency modulation	0.0046 ↑	0.1400	0.0287
Peak frequency modulation	0.0070 ↑	0.9321	0.2648

Table 3.8: The changes to call characteristics following denervation at P16.

P values from pre and post surgery comparisons shown and significant *p* values are highlighted in red. White arrows signify the direction in which the call characteristic changed after surgery. Begging calls remain unchanged in all of the call characteristics measured here following sham surgeries or right denervations. Left denervations cause significant increases in a broad set of entropy and frequency modulation measures.

The peak entropy of a call does not change following surgery. Presurgery ($M = -2.847$, $SD = 0.9381$) and postsurgery ($M = -2.743$, $SD = 0.9841$) calls are statistically similar, $t(9) = 1.212$, $p = 0.2602$ (Figure 3.9B).

Standard deviation of entropy within calls, which is a measure of how much variability in entropy there is within a call, does not change following surgery. Presurgery ($M = 0.8175$, $SD = 0.2683$) and postsurgery ($M = 0.8193$, $SD = 0.2753$) calls are statistically similar, $t(9) = 0.1091$, $p = 0.9158$ (Figure 3.9C).

Average frequency modulation does not change following a sham surgery. Presurgery ($M = 21.94$, $SD = 5.734$) and postsurgery ($M = 20.44$, $SD = 5.509$) calls are statistically similar, $t(9) = 1.638$, $p = 0.1400$ (Figure 3.9D).

The peak frequency modulation in a call does not change following surgery. Presurgery ($M = 84.84$, $SD = 2.171$) and postsurgery ($M = 84.79$, $SD = 1.460$) calls are statistically similar, $t(9) = 0.08796$, $p = 0.9321$ (Figure 3.9E).

Right denervations

Average call duration does not change following surgery. Presurgery ($M = 221.3$ ms, $SD = 59.18$ ms) and postsurgery ($M = 197.6$ ms, $SD = 55.34$ ms) calls are statistically similar in duration, $t(12) = 1.168$, $p = 0.2809$.

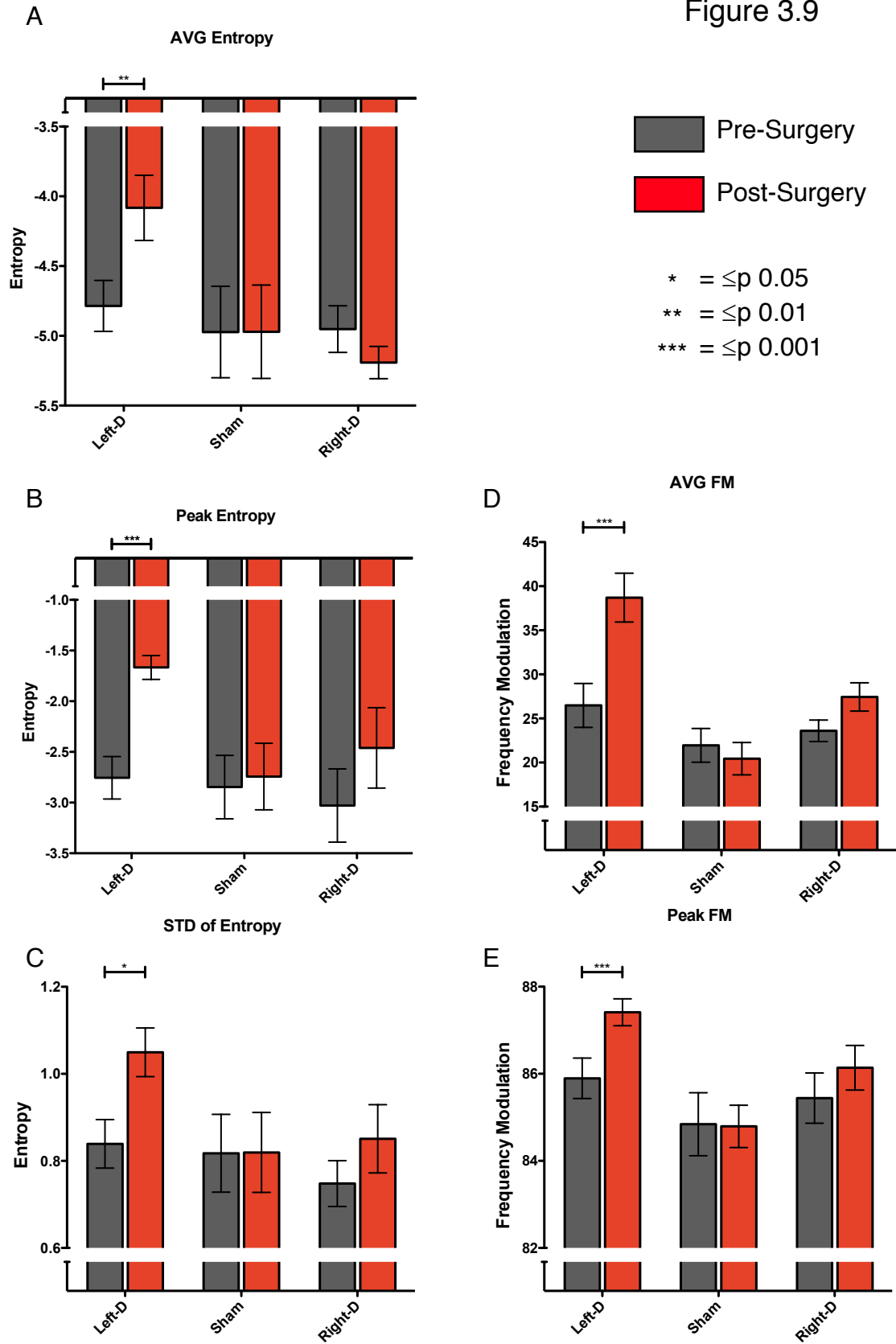
Figure 3.9: At P16, the call characteristics of food-begging calls change significantly with left, but not right or sham, denervations. Call

characteristics before and after left, right, or sham denervation surgeries.

Average Entropy (**A**), Peak Entropy (**B**), standard deviation of variance within calls (**C**) increases after left-denervation. STD = Standard deviation. Left-denervation also causes an increase in frequency modulation (**D**) and in peak frequency modulation (**E**) in food-begging calls. Neither sham nor right-denervations caused significant changes in any call characteristics above.

Averages \pm Standard error shown.

Figure 3.9



Average call frequency does not change following surgery. Presurgery ($M = 6387$ Hz, $SD = 319.5$ Hz) and postsurgery ($M = 6180$ Hz, $SD = 462.7$ Hz) calls are statistically similar in frequency, $t(12) = 1.765$, $p = 0.1081$.

Average call entropy does not change following surgery. Presurgery ($M = -4.952$, $SD = 0.5793$) and postsurgery ($M = -5.191$, $SD = 0.3994$) calls are statistically similar, $t(12) = 1.788$, $p = 0.1014$ (Figure 3.9A).

The peak entropy of a call does not change after right denervations. Presurgery ($M = -3.029$, $SD = 1.249$) and postsurgery ($M = -2.460$, $SD = 1.373$) calls are statistically similar, $t(12) = 2.972$, $p = 0.0227$ (Figure 3.9B).

Standard deviation of entropy within calls, which is a measure of how much variability in entropy there is within a call, does not change following surgery. Presurgery ($M = 0.7479$, $SD = 0.1823$) and postsurgery ($M = 0.8509$, $SD = 0.217$) calls are statistically similar, $t(12) = 1.356$, $p = 0.2024$ (Figure 3.9C).

The peak frequency modulation in a call does not change following surgery. Presurgery ($M = 85.44$, $SD = 2.000$) and postsurgery ($M = 86.14$, $SD = 1.774$) calls are statistically similar, $t(12) = 1.175$, $p = 0.2648$ (Figure 3.9D).

Average frequency modulation does not change following a sham surgery.

Presurgery ($M = 23.61$, $SD = 4.226$) and postsurgery ($M = 27.44$, $SD = 5.555$) calls are statistically similar, $t(12) = 2.515$, $p = 0.0287$ (Figure 3.9E).

Left denervations

Average call duration does not change following surgery. Presurgery ($M = 210.7$ ms, $SD = 62.60$ ms) and postsurgery ($M = 221.0$ ms, $SD = 83.69$ ms) calls are statistically similar in duration, $t(12) = 0.6791$, $p = 0.5111$.

Average call frequency does not change following surgery. Presurgery ($M = 6312$ Hz, $SD = 549.2$ Hz) and postsurgery ($M = 6508$ Hz, $SD = 690.2$ Hz) calls are statistically similar in frequency, $t(12) = 1.107$, $p = 0.2899$.

Average call entropy increases following left-denervation. Presurgery ($M = -4.785$, $SD = 0.6574$) and postsurgery ($M = -4.083$, $SD = 0.8425$) calls are statistically different, $t(13) = 3.052$, $p = 0.0100$ (Figure 3.9A).

The peak entropy of a call increases after left-denervation. Presurgery ($M = -2.755$, $SD = 0.7256$) and postsurgery ($M = -1.667$, $SD = 0.4089$) calls are statistically different, $t(12) = 5.514$, $p = 0.0002$ (Figure 3.9C).

Standard deviation of entropy within calls, which is a measure of how much variability in entropy there is within a call, increases following surgery. Presurgery

($M = 0.8392$, $SD = 0.1927$) and postsurgery ($M = 1.049$, $SD = 0.1936$) calls are statistically different, $t(12) = 2.839$, $p = 0.0160$ (Figure 3.9E).

Average frequency modulation increases following left denervation. Presurgery ($M = 26.48$, $SD = 8.613$) and postsurgery ($M = 38.71$, $SD = 9.596$) calls are statistically different, $t(12) = 3.542$, $p = 0.0046$ (Figure 3.9B).

The peak frequency modulation in a call increases after left denervation. Presurgery ($M = 85.90$, $SD = 1.608$) and postsurgery ($M = 87.41$, $SD = 1.069$) calls are statistically different, $t(12) = 3.306$, $p = 0.0070$ (Figure 3.9D).

Notably, the data of left denervated birds presented in Figure 3.9 was re-averaged for male ($n = 6$) and female ($n = 6$) nestlings to assess whether there were any sex differences. For each surgery type (L, R, Sham) within each call characteristic, A Two-Way Mixed-Factors ANOVA was carried out with sex (M, F) as one level and surgery outcome (Pre and Post) as another. As expected from the sonogram data, none of the pre or post denervation measures were significantly different between males and females (data not shown).

Average entropy, peak entropy, and the variance of entropy in begging calls significantly rose in left-denervated birds (Figure 3.9A - C). The entropy measures indicate that the calls of left denervated birds become on average 'noisier' (less like pure tones) throughout the call, that the noisiest peak in the call

was higher, and the noisiness of the call varied more throughout the call. Moreover, average FM as well as peak FM selectively increased in left-denervated birds (Figure 3.9D, E), indicating that the frequency produced during begging changes much more throughout the call in left-denervated birds. Changes in both of these general call characteristics are perhaps somewhat unsurprising given that one of the visually distinctive features of left-denervations at P16 is the appearance of noisy, highly fragmented ‘runs’ in the begging call (Figure 3.4, yellow underlines). Still, these quantifications support our earlier interpretations of sonogram data, that only left denervations severely affect the fine spectral structure of begging calls. Interestingly, the average call duration did not change following any Ts denervation surgery (Table 3.8). However, the variability of call duration, as reflected in standard deviation values, rose substantially (33.7%) after left denervation only, suggesting that some features of begging calls may remain relatively unchanged but become less stereotyped following left denervation of the Ts nerve.

P15 denervations:

The same procedures for handling data and analysis as outlined for P16 denervations were carried out in birds denervated when one day younger. Pre-surgery calls were all the calls recorded at P14 and all post-surgery calls those recorded at P15. Left-denervations (n = 6), Right-denervations (n = 6), Sham-denervations (n = 6) occurred at night.

Results:

No surgery type (L, R, Sham) at P15 recapitulated the changes to entropy or frequency modulation from P16 denervations. Interestingly, right denervations caused a significant decrease in entropy, which aligns with the much simplified calls seen post-denervation (Figure 3.7). Table 3.10 summarizes all of the results found in detail below.

Sham denervations

Average call entropy does not change following surgery. Presurgery ($M = -5.277$, $SD = 0.189$) and postsurgery ($M = -5.114$, $SD = 0.298$) calls are statistically similar, $t(6) = 2.355$, $p = 0.0999$ (Figure 3.11A).

The peak entropy of a call does not change following surgery. Presurgery ($M = -3.468$, $SD = 0.3377$) and postsurgery ($M = -3.201$, $SD = 0.6323$) calls are statistically similar, $t(6) = 1.316$, $p = 0.2798$ (Figure 3.11B).

Standard deviation of entropy within calls, which is a measure of how much variability in entropy there is within a call, does not change following surgery. Presurgery ($M = 7.234$, $SD = 0.9275$) and postsurgery ($M = 7.543$, $SD = 0.9826$) calls are statistically similar, $t(6) = 1.461$, $p = 0.2400$ (Figure 3.11C).

Average frequency modulation does not change following a sham surgery.

Presurgery ($M = 7.234$, $SD = 0.9275$) and postsurgery ($M = 7.543$, $SD = 0.9826$) calls are statistically similar, $t(6) = 1.461$, $p = 0.2400$ (Figure 3.11D).

The peak frequency modulation in a call does not change following surgery.

Presurgery ($M = 73.38$, $SD = 0.8744$) and postsurgery ($M = 73.55$, $SD = 3.792$) calls are statistically similar, $t(6) = 0.1131$, $p = 0.9171$ (Figure 3.11E).

Right denervations

Average call entropy decreases following right denervations. Presurgery ($M = -5.021$, $SD = 0.2288$) and postsurgery ($M = -5.299$, $SD = 0.2538$) calls are statistically different, $t(6) = 5.320$, $p = 0.0130$ (Figure 3.11A).

The peak entropy of a call does not change following surgery. Presurgery ($M = -3.147$, $SD = 0.7283$) and postsurgery ($M = -2.873$, $SD = 0.9092$) calls are statistically similar, $t(6) = 0.9427$, $p = 0.4154$ (Figure 3.11B).

Standard deviation of entropy within calls, which is a measure of how much variability in entropy there is within a call, does not change following surgery. Presurgery ($M = 0.7093$, $SD = 0.2296$) and postsurgery ($M = 0.8586$, $SD = 0.3511$) calls are statistically similar, $t(6) = 1.414$, $p = 0.2522$ (Figure 3.11C).

Table 3.10

Call Characteristic	Left Denervation	Sham	Right Denervation
Average entropy	0.3093	0.0999	0.0130 ↓
Peak entropy	0.1686	0.2798	0.4154
Standard deviation of entropy	0.2019	0.2400	0.2522
Average frequency modulation	0.2048	0.2400	0.5280
Peak frequency modulation	0.1519	0.9171	0.1515

Table 3.10: The changes to call characteristics following denervation at P15.

P values from pre and post surgery comparisons shown and significant *p* values are highlighted in red. White arrows signify the direction in which the call characteristic changed after surgery. Begging calls remain unchanged in all of the call characteristics measured here following sham surgeries or left denervations. The lack of changes after left denervation at P15 is in stark contrast to the changes that occur with the same surgery at P16. Right denervations have a significant *drop* in entropy, meaning that the calls become less noisy and more pure-tone-like. This change in entropy is in the opposite direction that denervations cause at P16 (Table 3.8).

Average frequency modulation does not change following a sham surgery.

Presurgery ($M = 10.50$, $SD = 4.509$) and postsurgery ($M = 9.481$, $SD = 2.487$) calls are statistically similar, $t(6) = 0.7118$, $p = 0.5280$ (Figure 3.11D).

The peak frequency modulation in a call does not change following surgery.

Presurgery ($M = 71.92$, $SD = 9.619$) and postsurgery ($M = 76.98$, $SD = 8.084$) calls are statistically similar, $t(6) = 1.914$, $p = 0.1515$ (Figure 3.11E).

Left denervations

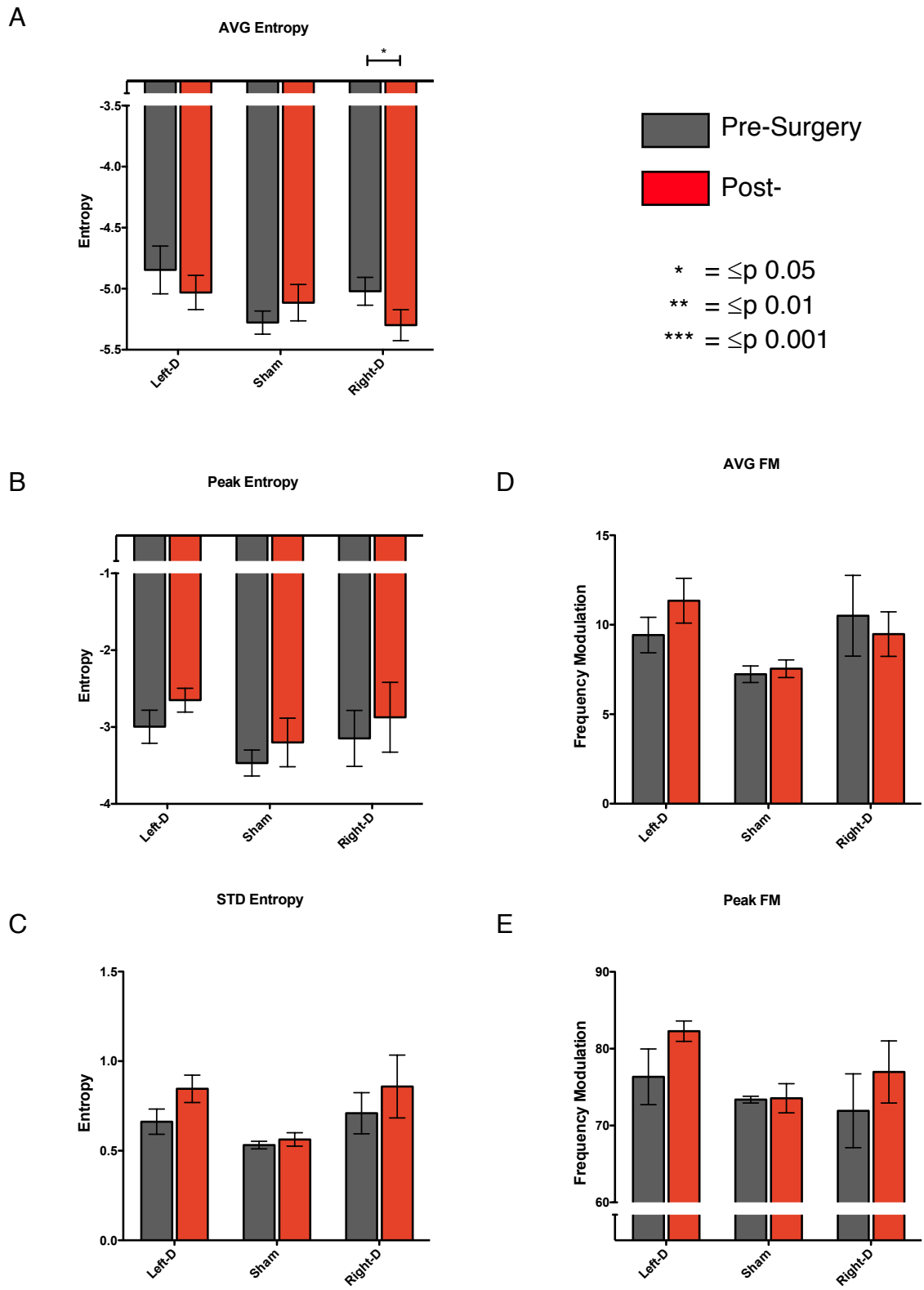
Average call entropy does not change following surgery. Presurgery ($M = -4.846$, $SD = 0.4796$) and postsurgery ($M = -5.031$, $SD = 0.3442$) calls are statistically similar, $t(6) = 1.131$, $p = 0.3093$ (Figure 3.11A).

The peak entropy of a call does not change following surgery. Presurgery ($M = -2.996$, $SD = 0.5272$) and postsurgery ($M = -2.650$, $SD = 0.3812$) calls are statistically similar, $t(6) = 1.609$, $p = 0.1686$ (Figure 3.11B).

Standard deviation of entropy within calls, which is a measure of how much variability in entropy there is within a call, does not change following surgery. Presurgery ($M = 0.6623$, $SD = 0.1721$) and postsurgery ($M = 0.8458$, $SD = 0.1872$) calls are statistically similar, $t(6) = 1.468$, $p = 0.2019$ (Figure 3.11C).

Figure 3.11: At P15, Neither left nor right denervation significantly change the call characteristics of food-begging calls. Call characteristics before and after left, right, or sham denervation surgeries. Neither sham nor unilateral Ts nerve sections cause significant changes in any call characteristics above. **A)** Average Entropy, **B)** Peak entropy, **C)** Standard deviation of entropy within calls. STD = Standard deviation. **D)** Frequency modulation, or **E)** Peak frequency modulation within calls. Averages \pm Standard error shown.

Figure 3.11



Average frequency modulation does not change following a sham surgery.

Presurgery ($M = 9.424$, $SD = 2.436$) and postsurgery ($M = 11.34$, $SD = 3.064$) calls are statistically similar, $t(6) = 1.4557$, $p = 0.2048$ (Figure 3.11D).

The peak frequency modulation in a call does not change following surgery.

Presurgery ($M = 76.34$, $SD = 8.876$) and postsurgery ($M = 82.29$, $SD = 3.251$) calls are statistically similar, $t(6) = 1.690$, $p = 0.1519$ (Figure 3.11E).

These results directly contrast those found in P16 denervated birds (Figure 3.8, 3.9). While left-denervations caused dramatic changes to the entropy and frequency modulation measures of begging calls at P16, they did not at P15. Thus, the fine analysis of call features support the gross sudden appearance of left-denervation effects at P16 that we saw in the sonogram data (Figure 3.4 versus 3.6).

Study 2C: Are the effects of left denervation due to our surgery paradigm?

The results of the denervation surgeries thus far presented have all come from individuals sampled at relatively long time intervals. Presurgery recordings occurred at ~1600 and post-surgery recordings were obtained at ~0600 and therefore roughly 10 hours passed between recordings, during which many changes in the call features of begging call may occur (Figure 2.3), potentially complicating the results of the denervation effects.

In order to test whether I could recreate the dramatic effects in left denervation effects at P16 using a more rapid protocol, 24 birds were split into one of 6 groups, a 2 x 3 experimental design of 2 ages (P15, P16) by 3 denervation groups (L, R, Sham). The birds were fed and recorded as normal. Two hours after a midday feeding (between 1100 - 1400), birds were anesthetized using 1.5 - 2% isoflurane in oxygen actively given into the oral cavity, which resulted in nestlings becoming unresponsive to a toe-pinch within 3 minutes and to be responsive and show no anesthesia effects within 10 minutes of removing isoflurane. Birds were anesthetized, unilaterally denervated, and returned to the nest showing no anesthesia effects within 30 minutes of removal from the nest. Birds were then fed and recorded within 5 minutes. Thus, using isoflurane, I was able to perform a denervation surgery on a nestling between normal feeding periods in the middle of the day, presumably causing less disturbance than denervations at night. Importantly, this allowed me to assess the effects of denervation within 30 minutes of the surgery.

Results and conclusion:

Denervating birds using a quicker, and potentially less disruptive protocol did not alter the results gained from overnight surgeries. Left tracheosyringeal (Ts) nerve section at P16 still resulted in a marked loss of begging call structure (Figure 3.12C). Neither right nor sham denervations had a severe effect on the

begging call (Figure 3.12A, B). Left, right, or sham denervations at P15 similarly had minimal effects (sonograms not shown).

In conclusion, strong asymmetric effects of denervation appear for the first time at P16. Right denervations at this age can result in the removal of a voice in begging vocalizations (Figure 3.5F, G), whereas left denervations have widespread effects, such that the entire structure of the begging call is altered. This effect is very reproducible (Figures 3.4) across two surgery paradigms (Figure 3.7). Furthermore, these results are supported by quantitative call analysis that shows clear effects of left, but not right or sham, denervations on begging call characteristics. The most curious finding was that the onset of left denervation effects was so sudden and reproducible at P16 across all the birds tested. In fact, no bird aged P15 or younger had its begging call so drastically altered as those in whom I sectioned the left Ts nerve at P16 ($n > 25$).

Experiment 3: Are A or B calls preferentially affected?

In chapter 2, I showed that later in begging call ontogeny, there are two call types produced concurrently, type A and type B calls (Figure 2.19). Intriguingly, the appearance of B calls at P16 (Figure 2.21), correlates with the time at which we first see strong left denervation effects (Figure 3.4). We thus wondered whether A and B calls were differentially affected by left denervation. Specifically, our right denervation data suggests that A calls appear to utilize both the left and right syringeal halves. Perhaps, however, the production of B calls is

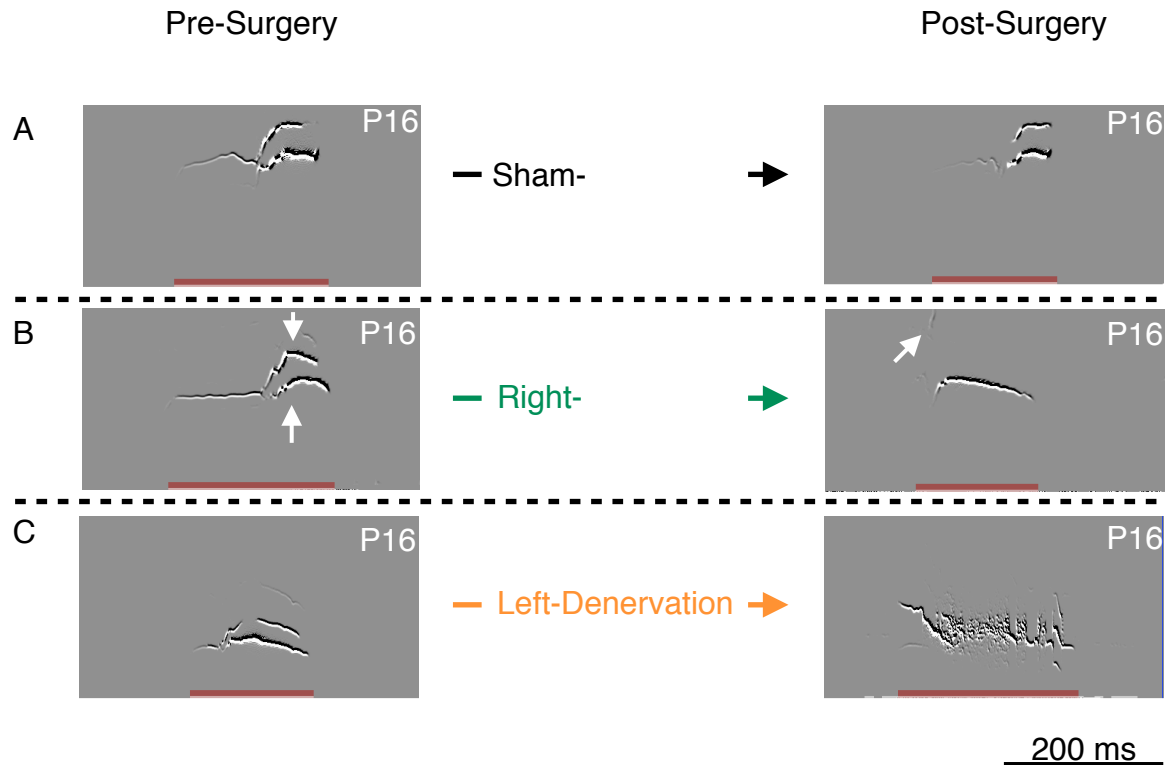


Figure 3.12: Denervation surgeries carried out on the same day produce similar results as overnight surgeries. Left, but not right or sham denervations at P16 caused large disorganizations of the begging call. Right denervation (**B**) resulted in the loss of one voice (white arrows) and the near disappearance of the first element of the **A** call, although note the faint high frequency sound where the first element temporarily resided. All pre-surgery sonograms are taken from the feeding preceding surgery. The post-surgery sonograms are taken within 2.5 hours of the presurgery sonograms.

left lateralized and their appearance at P16 accounts for the denervation phenotype we see.

The structure of A and B calls is distinctive enough (Figure 2.19) that I originally tried to perform call analysis by identifying A and B calls post-denervation. However, for almost all birds, the effects of left denervation were severe enough that this proved impossible (Figure 3.5). I next considered that the early B call approximates the structure of A calls with an added frequency modulated component (Figure 2.26) and thus the main difference in structure between the two calls might be the highly modulated portion of the call. Our rationale was that if the early B call has an A+B call structure and only one of the call types was affected, the resulting call would have one intact half and one disrupted half. Thus, I looked for vocalizations that appeared largely intact but with one portion of the call significantly affected. Again, while this approach might work in a subset of the calls of a small subset of animals (Figure 3.5D), it was not possible for the majority of birds (Figure 3.5).

Needing a new strategy, I recalled that B calls are preferentially produced at the beginning of begging bouts (Figure 2.20). Thus, if B calls are preferentially affected, the 1st call in a begging bout should have, for example, higher call entropy than the 5th, or vice versa. To undertake this analysis, each call of the first 8 - 13 begging bouts of 11 P16 left and 8 sham denervated birds was analyzed for call characteristics. A Two-Way ANOVA was carried out with surgery

type (Sham vs Left-denervation) as one level and call position (1 -5) as another. Tukey's Multiple Comparison Test was used to test for significance.

Results

Entropy (Figure 3.13A)

There was a statistically significant main effect of surgery type, $F(1, 20) = 7.486$, $p < 0.05$. There was also a main effect of bout position, $F(4, 20) = 4.157$, $p < 0.01$ and an interaction between surgery type and bout position, $F(4, 20) = 3.631$, $p < 0.01$. Post-hoc comparisons revealed that Left denervated birds had significantly higher entropy at every call position in the begging bout compared to sham individuals. Within sham birds, the 1st call in a begging bout had significantly higher entropy ($M = -4.075$, $SD = 0.454$) than the 2nd ($M = -4.405$, $SD = 0.611$, $p < 0.001$), 3rd ($M = -4.385$, $SD = 0.722$, $p < 0.001$), 4th ($M = -4.449$, $SD = 0.491$, $p < 0.001$), and 5th ($M = -4.330$, $SD = 0.594$, $p < 0.01$) calls in the begging bout. All other comparisons within sham birds were not significant. Within left denervated birds, all call positions were statistically similar.

Frequency Modulation (Figure 3.13B)

There was a statistically significant main effect of surgery type, $F(1, 20) = 12.86$, $p < 0.01$. There was no significant effect of bout position, $F(4, 20) = 0.6132$, $p > 0.05$ or an interaction between surgery type and bout position, $F(4, 20) = 2.16$, $p > 0.05$. Post-hoc comparisons revealed that Left denervated birds had significantly frequency modulation at every call position in the begging bout

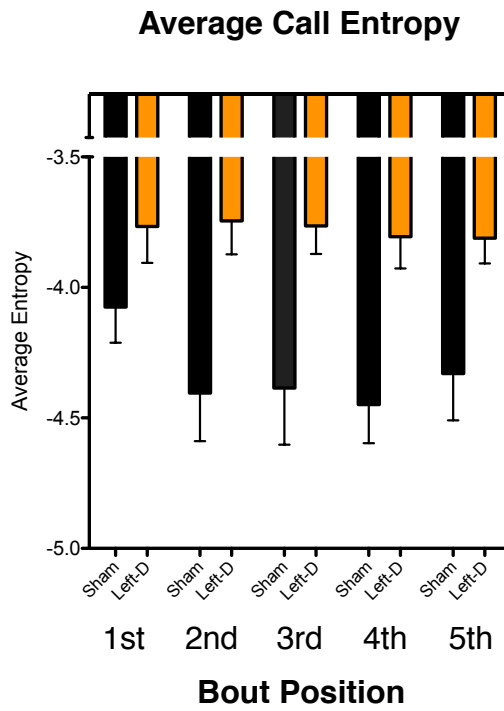
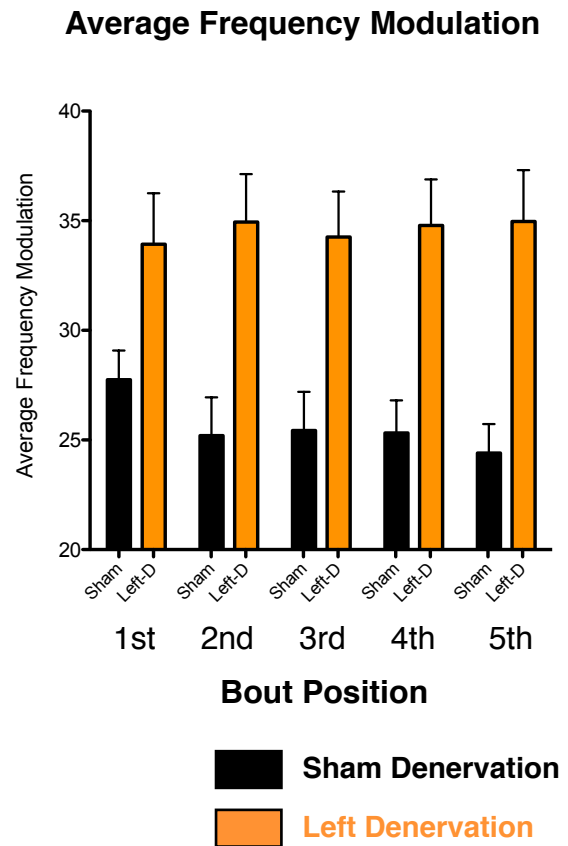
A**B**

Figure 3.13: Left denervation affects all calls. The average call entropy (**A**) and average frequency modulation (**B**) of begging calls in the first 5 positions of a begging bout after **sham** or **left denervation**. **A)** Left denervated fledglings have significantly higher call entropy than sham denervations at all call positions in a begging bout. Birds that received a sham surgery displayed significantly more call entropy in the 1st call position than all other call positions ($P < 0.05$). No such differences between calls was found in left denervated birds. **B)** Left denervated birds had significantly higher frequency modulation (FM) than sham denervated birds at every call position. Sham denervated birds displayed higher FM in the first call position than in the 5th ($p < 0.05$). Left denervated birds had no such differences between the calls.

compared to sham individuals. Within sham birds, the 1st call in a begging bout had significantly higher frequency modulation ($M = 27.747$, $SD = 4.424$) than the 5th call ($M = 24.403$, $SD = 4.384$, $p < 0.01$) in the begging bout. All other comparisons within sham birds were not significant. Within left denervated birds, all call positions were statistically similar.

Conclusions

To analyze whether A or B calls were preferentially affected by surgery, I analyzed effects on calls in different call positions in a begging bout. Analysis of the first five calls produced in a begging bout in sham denervated birds showed that there are changes in both average entropy (Figure 3.13A) and frequency modulation (Figure 3.13B) across the begging bout. This squares well with what we see in typical fledgling begging bouts (Figure 2.19), which are characterized by B calls appearing in the first positions. B calls have higher call entropy and frequency modulation than A calls (2.18), and thus the beginning of a begging bout was predicted to have higher values for both of these call features in sham birds. It did (Figure 3.13). Left denervated birds produced calls with higher call entropy and more frequency modulation than sham birds, as was expected from previous analysis (Figure 3.9). However, in left denervated birds, I saw no pattern at all to the data within the begging bout. All of the first five begging calls produced in begging bouts were equally affected across both measures (Figure 3.13). This result suggests that all calls, A and B, were substantially altered after left denervation. Of course, we might consider that left denervations cause for

birds to produce only a single call type. However, in a few birds, distinct A-like and B-like calls can be distinguished (Figure 3.5D), suggesting that this is not the case.

Experiment 4: Do the calls recover following unilateral denervation?

In canaries, left denervation of adults during periods of crystallized song production (the breeding season, spring and summer) results in the loss of the majority of song syllables (Nottebohm et al., 1979; Nottebohm & Nottebohm, 1976; Nottebohm et al., 1976). These effects remain during the duration of the breeding season but in the following year, song returns under control of the right syrinx, providing strong evidence for plasticity in the lateralized control of song (Nottebohm et al., 1979). This plasticity is also present early in life. Left denervations in the first weeks of life but not when song development is well underway results in right hypoglossal control of song, indicating that plasticity of peripheral control of song diminishes as the bird ages and, particularly, begins to learn song (Nottebohm et al., 1979). I thus sought to find whether this early plasticity was present in the asymmetric production of begging calls I observed with left denervations. Specifically, I asked:

- 1) Do left denervations a few days before P16 result in the compensation by the right side at P16?
- 2) Do left-denervated birds recover begging call structure after P16?

To understand if left denervations at P14 result in compensation by the right side when the bird reaches P16, I performed left denervations (n = 11), right denervations (n = 12) and sham surgeries (n = 10) on P13 birds using isoflurane and assessed the begging call at P16. To assess whether birds ever recover a more typical begging call structure following left denervation, I followed the birds until P22, when birds produce fewer calls per feeding session, and preferring sometimes to beg silently. The birds were continuously recorded from P13 - P22 and P14 onwards was considered post-denervation. The recordings of begging calls were treated and analyzed as in experiment 2B (See 'Recording Preparation' in Appendix 1, Figure 3.14). A minimum of 100 begging calls per day was set as the threshold to be included in the analysis. The calls were then analyzed for average call entropy and frequency modulation using a 2-WAY ANOVA with 'surgery type' (L, R, Sham) as one level and 'Age' (P14 - P22) as another level.

Results

Entropy (Figure 3.15A)

There was a statistically significant main effect of surgery type, $F(2, 30) = 230.6$, $p < 0.0001$. There was also a main effect of age, $F(8, 30) = 42.67$, $p < 0.0001$ and an interaction between surgery type and age, $F(16, 30) = 8.957$ $p < 0.0001$. Post-hoc comparisons revealed that left denervated birds had significantly higher call entropy than sham or right denervated birds at P16 ($p < 0.001$), P17 ($p < 0.001$), P18 ($p < 0.001$), P19 ($p < 0.001$), P20 ($p < 0.001$), P21 (p

<0.001), and P22 ($p < 0.001$). Sham and right denervated birds were statistically similar except at P22, in which right denervated birds had significantly less call entropy than sham birds ($p < 0.01$).

Frequency Modulation (Figure 3.15B)

There was a statistically significant main effect of surgery type, $F(2, 30) = 156.5$, $p < 0.0001$. There was also a main effect of age, $F(8, 30) = 21.08$, $p < 0.0001$ and an interaction between surgery type and age, $F(16, 30) = 5.754$, $p < 0.0001$. Post-hoc comparisons revealed that left denervated birds had significantly more frequency modulation than sham or right denervated birds at P16 ($p < 0.001$), P17 ($p < 0.001$), P18 ($p < 0.001$), P19 ($p < 0.001$), P20 ($p < 0.001$), P21 ($p < 0.001$), and P22 ($p < 0.001$). Sham and right denervated birds were statistically similar except at P22, in which right denervated birds had significantly less frequency modulation than sham birds ($p < 0.01$).

The begging calls of birds left-denervated at P13 still suffered from pockets of noisy vocalizations and aberrant sounds starting at P16 (Figure 3.14). Note also how the structure of the call is severely affected, with the call example shown being almost 2.5 times longer in duration than the calls at P13-P15. Studying the sonograms of the bird shown in figure 3.12, it is also clear that the begging call does recover a typical structure across development. This conclusion was supported by analysis of average entropy and frequency modulation, two features that I have previously shown significantly change with left denervations (Figure 3.9). Only left denervated birds produce begging calls of

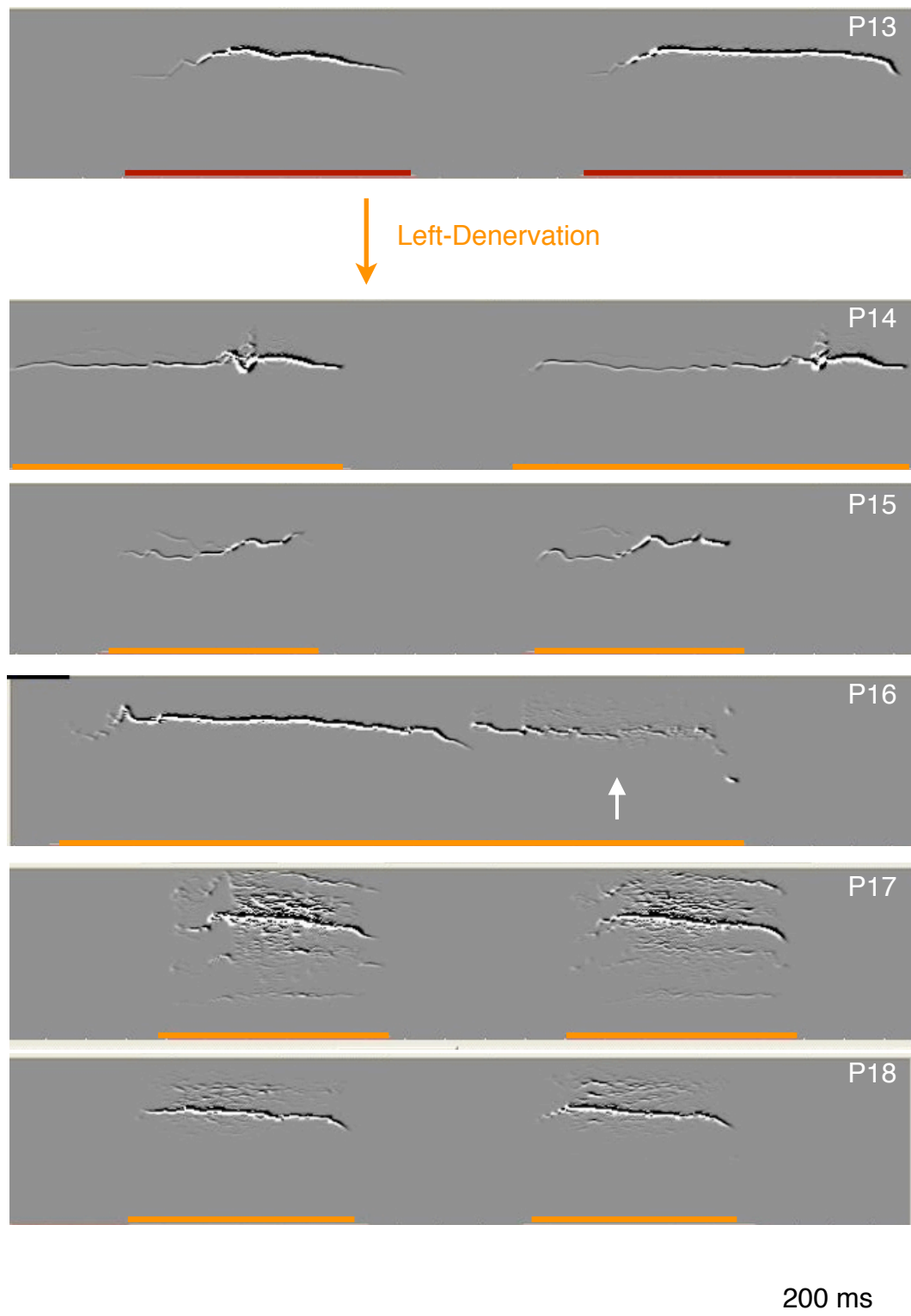
significantly higher entropy and with frequency modulation, and this effect arises robustly at P16 (Figure 3.14).

Experiment 5: Is the syrinx innervated ipsilaterally or bilaterally?

In a classic study describing some of the major song nuclei and their involvement in the production of song, Nottebohm, Stokes, and Leonard unilaterally denervated the syrinx in adult canaries and then stained for degenerating neurons using the Fink-Heimer technique. Their analysis revealed degenerating neurons in the ipsilateral n12, leading to the conclusion that in adult waterslager canaries, each syringeal half is innervated by the ipsilateral n12ts (Nottebohm et al., 1976). Further supporting this conclusion, unilateral denervations in adults results in marked muscular atrophy of the ipsilateral syringeal half (Nottebohm, 1971). In all of the denervation studies thus far presented, I made the assumption that in nestling canaries, n12 similarly innervates only the ipsilateral syrinx. However, considerable axonal remodeling occurs throughout the nervous system in development and some species of birds have bilateral projections to the syrinx from n12ts (Manogue & Nottebohm, 1982) and it thus became critical for the interpretation of our experiments to understand the innervation of each syringeal half.

Figure 3.14: The structure of food-begging calls begins to deteriorate at P16 in animals that were denervated at P14 and this effect remains. The food-begging calls of one individual from P13 - P18. The individual received a left-denervation at P13. The typical effects of left-denervation post P16 of noisy stretches in the call can be seen appearing in this individual at P16 (Arrow). The begging call remains noisy for the duration of the animal's begging vocalizations.

Figure 3.14



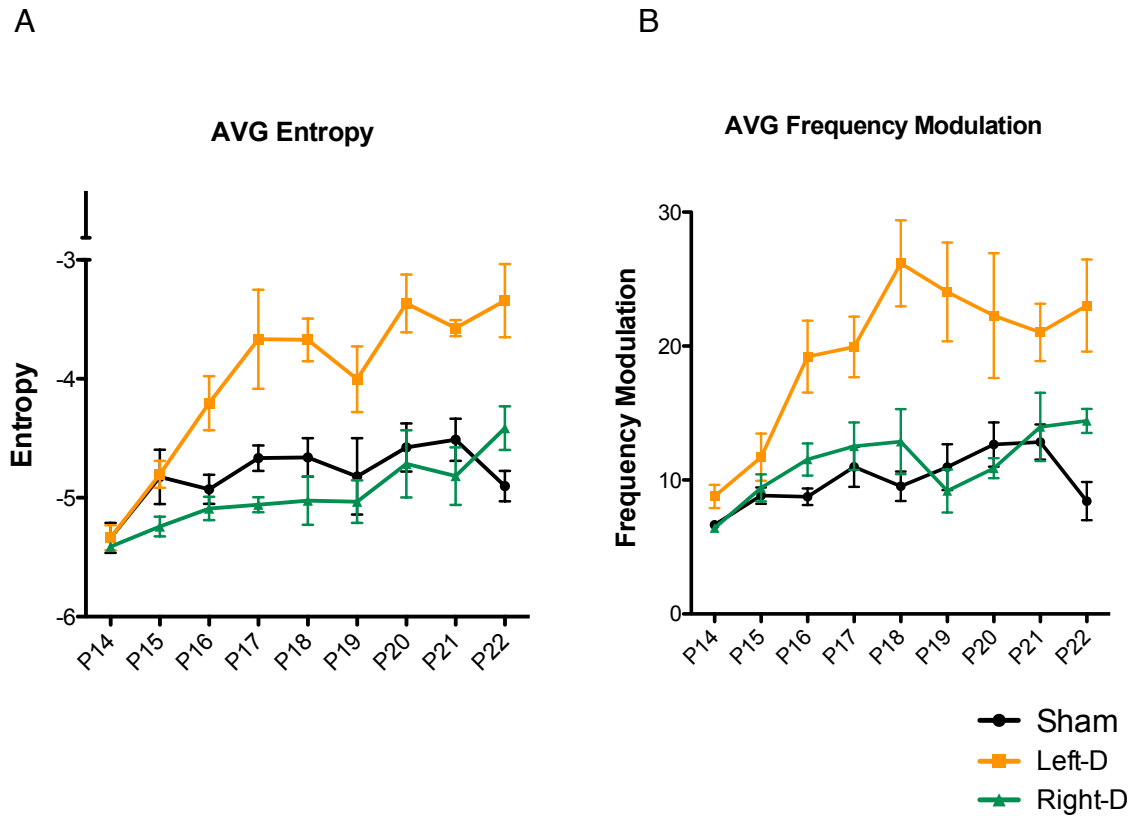


Figure 3.15: The effects of left-denervation appear at P16 and are long lasting. Average entropy (**A**) and frequency modulation (**B**) after left ($n=10$), right ($n=11$), or sham ($n=12$) denervation surgeries are displayed above. Note that the increases in average entropy and frequency modulation for left-denervation occur at P16 even in these birds which were denervated pre-P16, and that this effect remains. Only call analysis on post-denervation calls is shown above. Right and sham denervations were statistically similar across both call characteristics.

In order to map what nuclei innervated each syrinx, I used the Bartha strain of pseudorabies virus (PRV), a widely validated tool for tracing neural circuits (Ekstrand, Enquist, & Pomeranz, 2008; Perez et al., 2011; Pomeranz, Reynolds, & Hengartner, 2005; Smith et al., 2000). This attenuated virus propagates in a retrograde manner through chains of synaptically linked neurons. PRV has a ~24 hour cycle inside a cell during which the virus infects the axonal terminals of a neuron, moves to the cell soma, replicates, results in the cell producing -in the strain used in these studies- EGFP, moves to presynaptic targets and gets released, starting the infection cycle again in the next neuron. Thus, peripheral injections into musculature or organs can, by varying number of days allowed for infection, reveal the neuroanatomical circuits in a hierarchical manner (Figure 3.16).

The specific strain I used in these experiments was PRV-152, that constitutively expresses EGFP (Smith et al., 2000). The virus was generously prepared by Christian Perez, at the time a postdoctoral fellow in Jeff Friedman's lab, as described elsewhere (Perez et al., 2011; Smith et al., 2000) and was originally a kind gift by Lynn W. Enquist, Department of Molecular Biology, Princeton University.

To assess the nature of innervation of the syrinx by n12ts, I collected 26 canary nestlings (P5 - P8) from nests in our breeding colony and hand raised

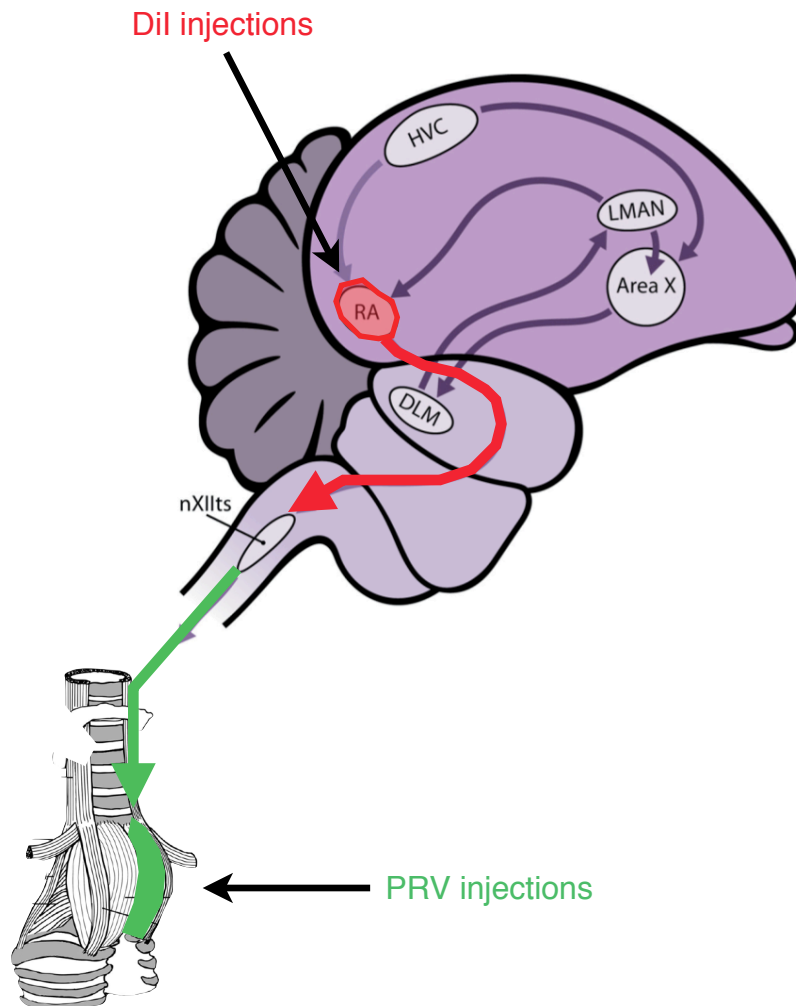


Figure 3.16: Schematic of Dil and PRV injections. **Red)** stereotactically guided Dil injections into nucleus RA should result in the anterograde labeling of nucleus n12. **Green)** Injections of high titer PRV into one half of the syrnix should result in the retrograde labeling of projection neurons, presumably in n12.

them throughout the experiment. I was specifically interested in understanding the nature of the innervation of the syrinx at P16 and whether this was potentially different at P15. I wanted to assay the innervation patterns of the syrinx and whether they possibly changed, say, from bilateral to unilateral across this time period. When birds reached either P14 or P15, at midday (1000 - 1400) and after a 2 hour food deprivation, they were anesthetized with 1:5 Nembutal at a dose of 5.7 μ l per gram of body weight, and allowed to lie until unreactive to a toe pinch. During this waiting period, a small pulled pipette needle was filled with 3 μ l of PRV-152 to ready it for later injection. When the bird was unreactive to a toe pinch or to feathers being plucked, the ventral neck feathers were removed to expose the clavicles. A V-shaped incision was made along the skin overlying the intraclavicular space and the fat inside this body cavity was lifted out, exposing the intraclavicular airsac. The airsac was punctured and moved aside to expose the thoracic cavity. The PRV-filled pipette was lowered through the interclavicular space by hand and 5-8 injections were made into either the left or right half of the ventral syringeal muscles (tracheobronchialis ventralis and the lateral portion of the syringealis ventralis; Figure 1.4). After each injection, the pipette was left in place for 30 seconds to allow liquid to dissipate within muscle fibers. Injection volumes were small enough that there were no visible drops or additional wetness on the surface of syringeal muscles following injections. Birds in which viral injections were mistargeted or virus-containing liquid dropped into the thoracic cavity were noted and not quantified. The fat that was previously removed from the interclavicular space was replaced and the skin sealed with

tissue adhesive and antibiotic (Neosporin ointment) was applied to the wound area. Birds were placed under a heat lamp to recover and later returned to the nest and fed as normal.

PRV-injected Birds were given 24 ± 1 or 48 ± 1 hours and then perfused (See 'Perfusion of Tissue' in Appendix 1). Both the brain and syrinx were collected and then sectioned in a cryostat (See 'Sectioning of perfused tissue' in Appendix 1). Frontal sections of n12ts through nucleus RA were investigated for EGFP-positive neurons.

Results

Injections of PRV into either the left or right syrinx resulted in EGFP neurons in only the ipsilateral n12ts, regardless of age tested (Figure 3.17). The successful infection of PRV and the unilateral targeting of injections were first verified by analyzing frontal sections of the syrinx. EGFP positive puncta in the syringeal muscles were present in every bird analyzed ($n = 19$), and only visible on the syringeal half that received injection (Figure 3.15A), indicating that the viral infection did not spread widely in muscle tissue. The number of EGFP expressing neurons found in n12ts after 24 hours was always low enough to be counted (a range of 3 - 21 neurons per bird in all samples analyzed, $n = 7$). No EGFP neurons were found frontal to n12ts, including in nucleus RA. After 48 hours, no EGFP positive neurons could be found in n12ts or any other brain region frontal to it, including RA ($n = 13$).

Conclusion

The syringeal muscles are innervated by the ipsilateral n12ts as early as P15. Though the *amount* of syringeal innervation may differ between ages as evidenced by the thickening of the syringeal nerve across nestling development (personal observation), the unilateral nature of syringeal innervation by n12ts does not appear to be different between P15 and P16 birds.

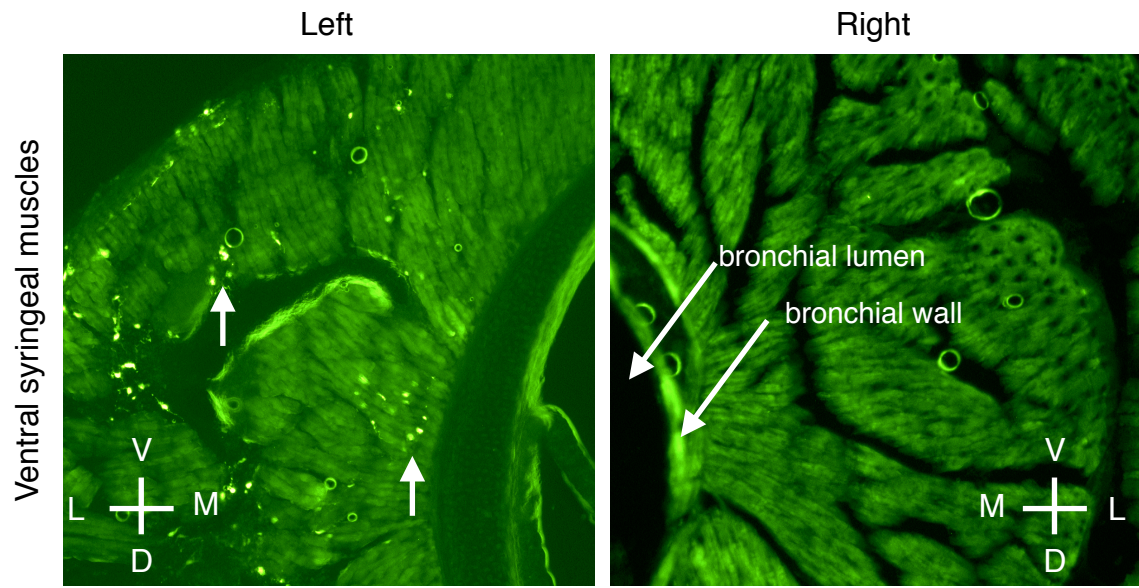
The multi-synaptic tracing of circuits using PRV has been done in a number of vertebrate species (Perez et al., 2011). In 13 canary nestlings, no EGFP expression could be found anywhere in the brain after 48 hours, including n12ts, a brain area that at 24 hours had EGFP positive neurons in every bird (n = 7) analyzed (Figure 3.17). In zebra finches, this observation was similarly made by Wanchun Liu in our laboratory and Erich Jarvis of Duke University (personal communications). In studies I carried out using PRV while a rotation student in Jeff Friedman's lab, motor nuclei of various cranial nerves in mice would express EGFP for 4 - 6 days after muscle injection, after which, neurons would die from infection. While we do not know why PRV-driven EGFP expression is not visible in the canary brain after 24 hours, there are likely two possibilities. Either the immune system of songbirds is particularly well-suited for warding off pseudorabies infection or the neurons die before the virus has a chance to cross synapses. In work using PRV injected into nucleus RA and retrogradely transported into HVC in adult zebra finches, Wanchun Liu found that a few days after injection, the song of birds was negatively affected (Personal

Figure 3.17: Each side of the syrinx is innervated by the ipsilateral n12.

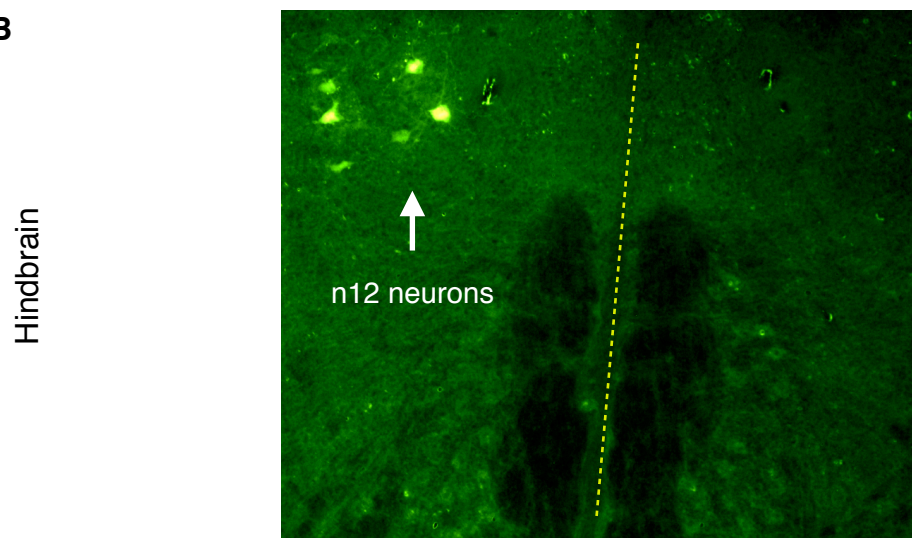
In the example above, Injections of PRV into the left ventral syringeal muscles resulted in EGFP expression only in these muscles (**A**; arrows). After 24 hours, GFP expression is clearly visible in large n12 neurons only in the left n12 (**B**). The yellow line highlights the midline. M = medial, L = lateral, V = ventral, D = dorsal.

Figure 3.17

A



B



communication). While speculative, this observation of degraded song following PRV infection suggests that PRV-infected neurons are not protected by a robust immune system but may instead die before synaptic transmission. While the rapid shut-down of PRV in the songbird brain is interesting and perhaps of particular note to other songbird researchers interested in tracing circuits using this virus, for my purposes, PRV experiments confirmed that unilateral denervations selectively silence the motor output of the ipsilateral n12ts, the major motor nucleus of each hemisphere, further buttressing the case that unilateral denervations at P16 reveal central asymmetries in the production of begging calls.

Experiment 6: What is the nature of RA projections in the nestling canary?

Nucleus RA, the major premotor output of the song system (Figure 1.7) sends its major efferents to nucleus n12ts and RAm in the hindbrain (Nottebohm et al., 1976; Wild, 1993a, 1993b) and DM in the midbrain (dorsomedial nucleus of the intercollicular complex; Wild, 1993b) and a very small amount of projections back up to HVC (Bauer et al., 2008). As mentioned earlier, lesions to RA in late begging development affected the begging calls of male zebra finches (Liu et al., 2009). Moreover, in budgerigars (parakeets), lesions to the nucleus analogous to RA also caused disruptions of the late begging call while lesions did not affect early begging vocalizations (Heaton & Brauth, 1999, 2000a, 2000b). Thus, we

asked whether RA might play a distinctive role in the lateralization phenotype we see in our begging canaries. If RA were involved, there are, of course, many ways in which nucleus RA could affect the onset of lateralization. We considered three possibilities. First, the onset of innervation of n12ts may be asymmetric between the hemispheres, with, for example, the left innervating the syrinx first and thereby taking a developmental lead which compounds over time with vocal practice. Second, innervation may occur at P16 but not before, and the new forebrain input causes lateralized effects. Third, the timing of innervation may play little or no role but the activity of nucleus RA drives lateralization in some way. To test the first two possibilities, I performed track-tracing experiments. To ask if nucleus RA might play a role in begging call lateralization, I first determined whether RA projects to n12ts at early ages.

I traced the projections of RA to n12ts using DiI (D383, Invitrogen), a long-chain diacylcarbocyanine that is widely used in the anterograde and retrograde tracing of living and fixed tissues. For all anterograde tracer surgeries, birds (P4 - P9) were removed from nests in our breeding colony and hand-raised throughout the entire experiment. When birds had reached the appropriate age, 2 hours after lights off, birds were removed from the nest and given a dose of 1:5 Nembutal at a dose of 5.7ul per gram of body weight. Once the bird was unreactive to a toe pinch, the bird was placed in a stereotaxic device and the feathers on the scalp removed to expose the underlying skin. A midline incision was made on the scalp and nucleus RA was located using stereotaxic coordinates. A small window was

made into the skull overlying nucleus RA and a small slit was made in the dura using a scalpel to expose the brain. A 30 μ glass micropipette that contained Dil was slowly lowered into the brain through the cranial window using stereotaxic equipment. Four injections were made per side at slightly different coordinates at 50nl per injection site. After each injection, I waited 1 minute to allow Dil to diffuse somewhat into the tissue before creating negative pressure by retracting the pipette. Afterwards, the skin was closed using tissue adhesive and antibiotic (Neosporin ointment) was applied to the wound area. The bird was placed under a heat lamp to aid recovery and, when nearly fully recovered, returned to the nest.

I performed unilateral Dil injections into nucleus RA in P11 - P14 nestlings and collected brain tissue after perfusions (See 'Perfusion of tissue' in Appendix 1) 40 - 48 hours after surgery, which preliminary experiments showed was sufficiently for robust signal to appear in the n12 of adult birds. Brain tissue was sectioned at 40 μ m in a cryostat at a frontal orientation.

Results and conclusions

Unilateral injections of Dil in a spring-time (singing) adult canary male result in strong ipsilateral signal of Dil in n12ts (Figure 3.18), replicating the conclusions of earlier work (Nottebohm et al., 1976). Unilateral injections into either the left or right nucleus RA of P11 - P14 canaries, and later collected when the individuals were P13 - P16 revealed similarly robust unilateral, but not

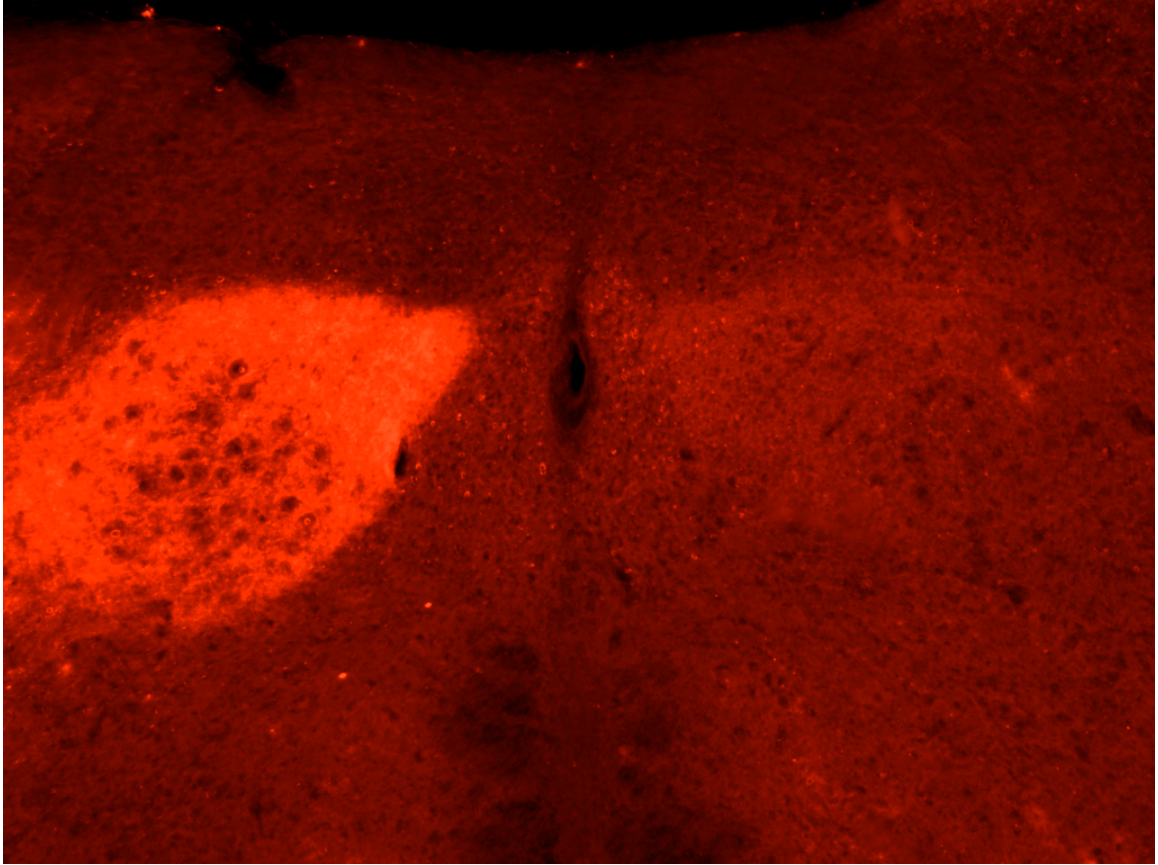


Figure 3.18: Injection of anterograde tracer into the left nucleus RA of adult canaries results in strong labeling of the left, but not right, n12.

Frontal section through nucleus n12, a tear-drop shaped motor nucleus that projects to the syrinx via the tracheosyringeal nerve.

contralateral, Dil signal (Figure 3.19). Misses to nucleus RA resulted in an outline-like effect, with Dil present all around, but not inside the nucleus (Not shown), which has been observed in both canaries and zebra finches with various neuronal tracers (Clare Walton, Wanchun Llu, Tony Lombardino, Eben Pariser, personal communications). Importantly, these nucleus RA misses resulted in no visible transportation of Dil into n12ts, demonstrating that the surrounding tissue of RA does not project to n12ts. Thus, the n12ts innervation patterns we see from our injections, even when depositing Dil just beyond RA, are specific to the projections emanating from RA. This study demonstrated that nucleus RA projects to nucleus n12ts as early as P13 and both RAs appear roughly symmetrical in their innervation of the nucleus. The Dil signal seen in n12ts is determined by a number of uncontrolled factors, including amount of Dil deposited within nucleus RA and the exact amount of hours after surgery before brains were collected. If there were large asymmetries in innervation such as one side arriving a day ahead of another for example, we would be able to detect them. Small asymmetries were not accounted for in these studies. The previous experiment utilizing PRV showed that n12ts→syrinx innervation is also ipsilateral in nature. Thus, collectively, these two track-tracing studies show that the major descending motor pathway for vocal production (RA→n12ts→syrinx) is contained ipsilaterally in nestling canaries.

Experiment 7: Is RA active in nestlings?

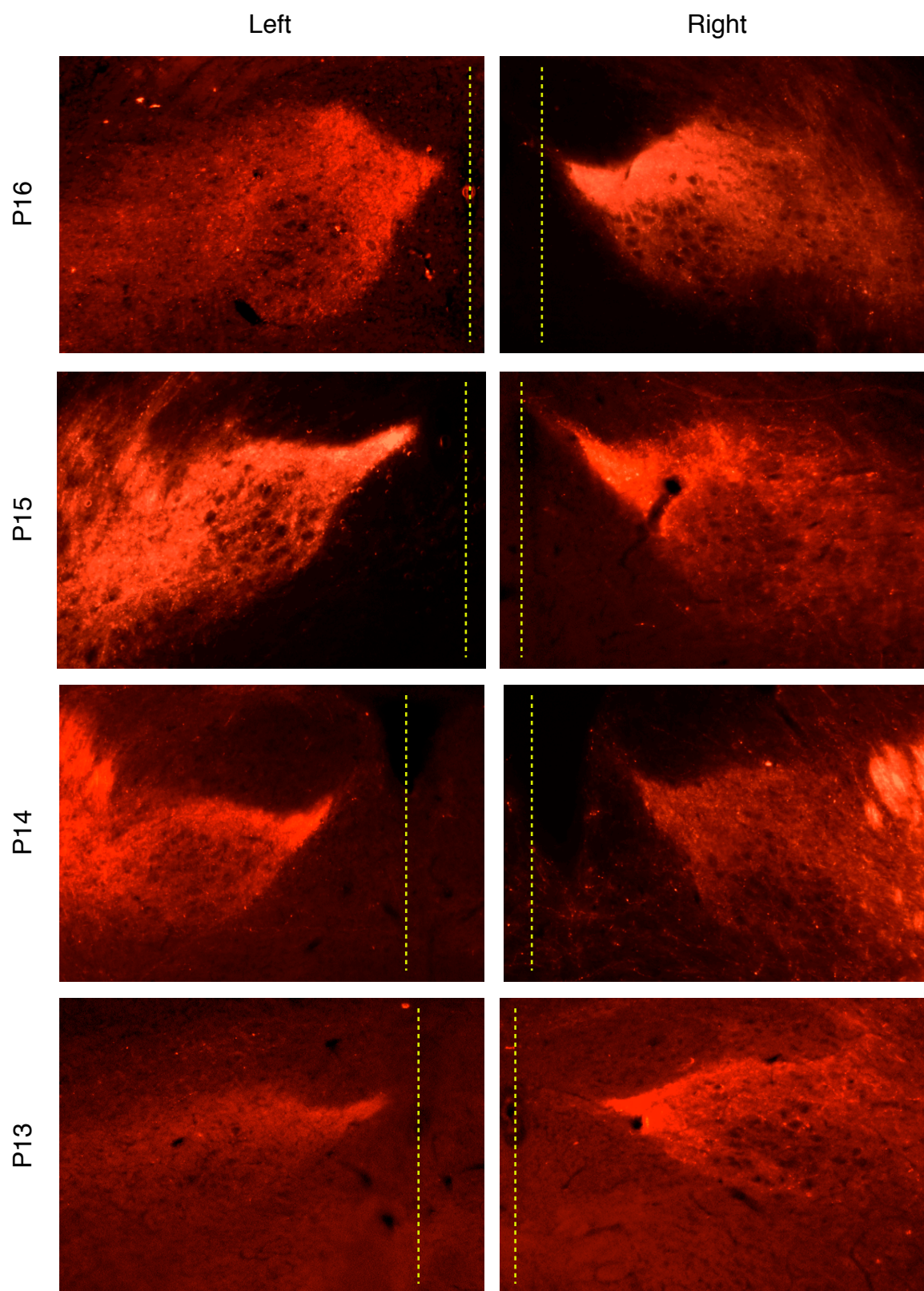
I used cytochrome oxidase (CO) histochemistry as an assessment of activity levels of nucleus RA in P15 - P16 canary nestlings. Cytochrome oxidase is the terminal enzyme in the respiratory electron transport chain of mitochondria and is used in the formation of ATP. Highly active brain areas, or those with higher metabolic needs, have higher concentrations of CO, making this technique a reliable indicator of neuronal activity (Adret & Margoliash, 2002; Reviewed in: Gonzalez-Lima, 1998). In zebra finches, cytochrome oxidase activity is very low in RA at P20 but rises sharply beginning at P30, correlating with an increase in RA firing rates (Adret & Margoliash, 2002). For my purposes, I wanted to assess 1) What CO activity patterns look like in the begging canary brain, 2) whether CO activity changed across the P15 - P16 transition and 3) whether there are left-right differences in CO activity in major song-related nuclei at any of the ages investigated.

Hatchling canaries (P4 - P9, total n = 12) were collected from nests within our breeding colony and hand reared for the duration of experiments. When birds reached appropriate age (P15 or P16 n = 6 each age), post-fixed in 4% PFA for 24 - 48 hours. For details on the methodology for cytochrome oxydase histochemistry, please see 'Cytochorome C' in Appendix 3. Briefly, perfused brains were mounted in 2.5% agarose and sectioned at 200µm in a vibratome, collected in 1X PBS and stored at 4°C until used for staining

Figure 3.19: Nucleus RA projects to the ipsilateral n12 as early as P13.

All birds shown above received unilateral injections of the anterograde tracer Dil into nucleus RA. As in the adult (Figure 3.16), Dil was only found in the ipsilateral n12. Each side of every age above was verified with at least 2 animals. Yellow bar highlights the midline.

Figure 3.19



(within 24 hours). Free floating sections were incubated in fresh 0.3% cytochrome C for 8 hours. Tissue from control and experimental animals were processed together in the same crucibles. Sections were rinsed in 1X PBS, mounted on slides using 0.3% gelatin, dehydrated through a series of increasing concentrations of ethanols and coverslipped to preserve tissue until analysis was undertaken.

For quantitative densitometry, images of brain sections containing regions of interest (RA, HVC, n12ts, and RAm) were captured using A Photometric Cool Snap Cf CCD camera with a Nikon lens (AF Micro NikkoR, 60 mm, 1:2.8D) and MCID Elite 6.0 (Rev 1.0) software. The mean gray value (of 256 gray levels) for each selected nuclei of interest was determined using MCID software. To compensate for background for background staining and control for variations in illumination level between images, the average pixel density for the lightest region within each section (Figure 3.20, red circles) was subtracted. To account for staining variation due to length of time incubated, length of time from sectioning to staining, and perfusion quality affecting permeability of tissue, all of the data are presented as a ratio of the optical density of each nucleus of interest divided by the optical density of lightly stained surrounding brain tissue = $OD_{\text{Nucleus of interest}} / OD_{\text{brain}}$.

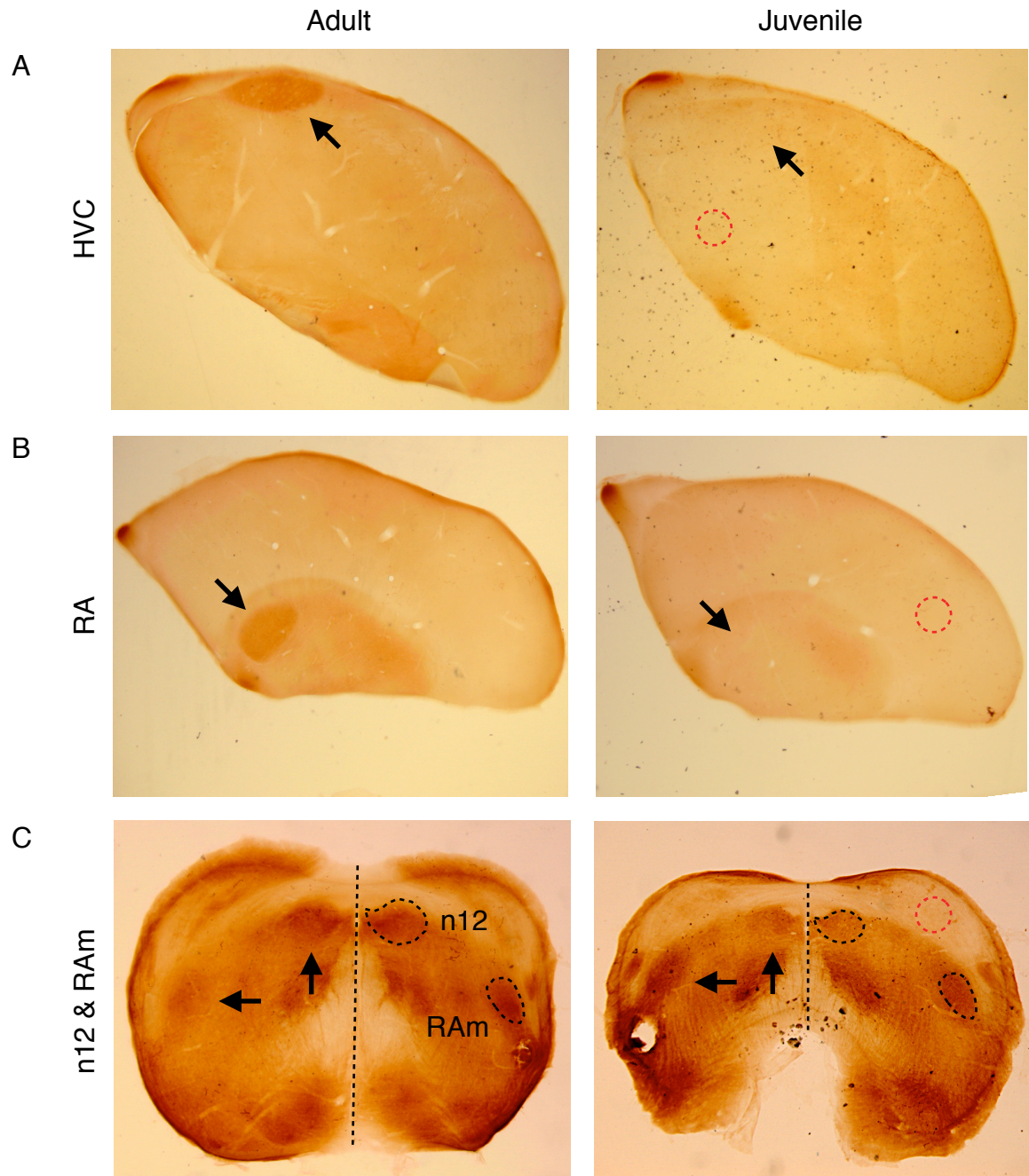
Results and conclusions

In adult male canaries, HVC, nucleus RA, n12ts, and RAm all showed significantly elevated CO staining relative to the surrounding tissue (Figures 3.20, 3.21). Juveniles, regardless of age, showed adult-like levels of CO staining relative to surrounding tissue in n12ts and RAm, but not in HVC or RA (Figures 3.20, 3.21). In fact, not a single animal showed any clearly distinguishable signal in HVC or nucleus RA. We wondered if perhaps the signal was too faint to be detected with our 8 hour incubation in 0.3% cytochrome C and we thus tried up to 40 hours in 3 P17 canary fledglings with no success in delineating forebrain nuclei from the surrounding tissue. There were no statistical differences in the optical density between left and right n12ts or RAm (data not shown).

In agreement with a previous report in zebra finches (Adret & Margoliash, 2002), no nucleus RA signal was found in my fledgling canaries. Additionally, in P15 - P16 canary nestlings, significant CO signal can also not be found in HVC, but n12ts and RAm can be easily picked out from the surrounding neuropil. Thus, there are two main conclusions from these data. First, cytochrome oxydase staining supports the interpretation of denervation data showing that nucleus n12ts is active across P14 - P17 in the production of begging calls (Figures 3.4 - 3.7). Moreover, no differences between the left or right n12ts (or RAm) were detected, suggesting that each side may be similarly mature or active. Second, no CO signal was found in song system motor nuclei in the forebrain. While this does not mean that RA does not play a role in the production of begging calls, it

Figure 3.20: In P15 - P17 canaries, neither nucleus RA nor HVC is positive for cytochrome oxidase staining but n12 and RAm are. All tissue displayed above are of frontal sections. Cytochrome oxidase staining in spring-time male adults revealed strong signal in HVC (A), RA (B), n12, and RAm, a respiratory nucleus (C; arrows). On the other hand, P15 - P17 canaries did only had distinguishable signal in n12 and RAm (C). In the C panels, the right half of the hindbrain is used to outline the nuclei to aid in identification. Black dotted line highlights the midline. The red circles display areas of tissue that were used to calibrate measurements. All sections within a column are taken from the same animal. The juvenile pictured here was P16.

Figure 3.20



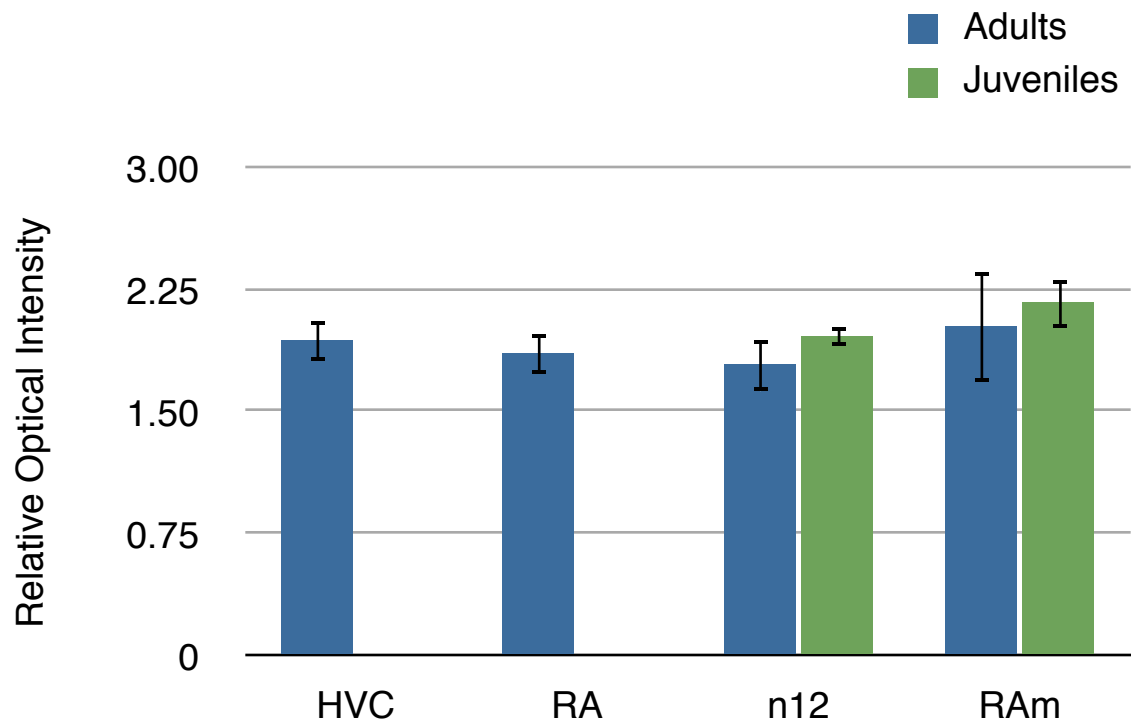


Figure 3.21: Relative optical intensity of cytochrome oxidase staining in various brain nuclei. In spring-time adult male canaries, HVC, nucleus RA, n12 and RAm have significantly higher levels of cytochrome oxidase staining than background. Only n12 and RAm show any staining above background in juvenile birds (P15 -P17). There were no differences in any age studied and no juvenile displayed a single section with HVC or RA positive staining.

is clear that RA is not metabolically mature at this age in canaries, as in zebra finches (Adret & Margoliash, 2002).

Final thoughts

I have here presented a wide variety of experiments which all point to a single day in development as the day when lateralized vocal production can first be detected in canaries. Denervating the left, but not the right, Ts nerve causes significant disruptions of the calls at P16 and not earlier (Figures 3.1 - 3.7). This asymmetric effect of denervations at P16 is found whether I use different surgery paradigms (Figure 3.12), or denervate the birds two days ahead of time (Figure 3.15). Left denervation appears to affect all begging calls (Figure 3.5, 3.13) across a variety of call features (Figure 3.9). I have further shown that in my young canaries the descending motor pathway (RA→n12ts→syrinx) projects ipsilaterally (Figures 3.17, 3.19), supporting the interpretation that unilateral denervation may assay unihemisphere function (F., 1972; Nottebohm, 1971; Nottebohm & Nottebohm, 1976; Nottebohm et al., 1976).

An aspect of the data I present here that I find rather wonderful is that the lateralization of vocal production, which I have peeked into by utilizing denervations, would be so rigidly programmed to appear at P16. It has surely occurred to the careful reader that P16 is not a random day in the life history of a canary, but is the day these nestlings take their first major step towards

independence, namely, the fledge (Figure 2.23). Moreover, P16 is when canaries begin to produce a new call that may aid in communicating their location (Figure 2.21) or perhaps identity (Figure 2.25). Either way, it is a brave world they have stepped into and it is curious that they would drag vocal asymmetry with them. I will explore the role of fledging and asymmetric vocal production in my next chapter.

How Chapter 4 came to be

This chapter that follows happened by chance. In the Fall of 2009, my friends and I attended a risotto competition in the Lower East Side. At the time, I was in the middle of hand raising over 15 canaries and only gave myself two hours of risotto indulgence before I was due back for the next canary recording. Well, what was supposed to be 2 hours of risotto tastings turned into 5 hours and when I returned, with a guilty heart but a very satisfied belly, I found my young canaries utterly transformed. The calls became strange sounding and some birds stopped vocalizing during food begging altogether, preferring to back up, head down, beak closed while producing the strange vocalization at the presentation of food. I had no idea what to make of it so I scrapped the experiment and wrote what I saw off as strange.

What is so unique about being 16?

It is hard to miss that in the last two chapters one day in the early development of canaries continues to be of some note: post hatch day 16. In chapter 2, I described that canaries predominantly fledge at P16 and that B calls, a mechanically and communicative distinct call, also appears at P16. In chapter 3, hardly a page goes by without me mentioning this day as this is when denervations of the left, but not right, tracheosyringeal nerve cause havoc on the structure of the call. As all of these lines of experimentation began to merge around P16, I wondered why I saw so many changes at this time. Fledging was

clearly the frontrunner as the primary mover that could explain the emergence of a new call that perhaps conveyed novel communicative information such as location. Furthermore, while we do not know biologically what causes fledging, hormones might be a good bet and thus perhaps the hormone-related activity at this time aided in lateralizing a call that was previously not. Alternatively, the appearance of B calls, the asymmetric effects of left denervation, and fledging might all have nothing to do with one another and simply overlap temporally. When so many events change simultaneously, it is hard to know if one causes the others or if they are all caused by something altogether different. I began to wonder about how I might be able to disentangle fledging and B calls and left denervation effects from one another. And then I remembered the risotto effect.

Chapter 4: Hunger stress and the begging call

Accidental experiments had suggested that starved animals could produce B calls as early as P13. Using this potential new behavioral manipulation of withholding food during the day, I sought to understand whether these three P16-related behaviors I saw (B call appearance, fledging, asymmetric effects of denervations) always came together or could be disentangled.

Experiment 1: What are the effects of hunger stress on P16 canaries?

To test whether the appearance of B calls, fledging, and the asymmetric effects of denervation occurred coincidentally or were linked, 6 canary hatchlings (3 females, 3 males) were removed from nests at P6 - P8 from our breeding colony and hand-raised for the duration of the experiment. When the birds had reached P16, I food deprived them for 8 hours, from 0900 - 1700 (the birds were on a 14:10 light/dark cycle and thus this block of time of not being fed represented ~57% of their day). Each individual was fed at the '0hr' timepoint (0900) and pseudofed 2hr, 4hr, 6hr, and 8hr later, which entailed the presentation of food and induction of food begging calls but no actual feeding. The food syringe was placed into the mouth of the canary every few begging calls as was customary but no food was deposited. The very last time I inserted the food syringe in a feeding session I gave each bird a tiny amount (~0.5ml) of food so that the birds would continue to associate me with food: not enough to spoil them

but just enough to keep them coming back -the “casino effect”. This pseudofeeding protocol was the same for all time-points after 0hr (2hr, 4hr, 6hr, 8hr). The birds were video-recorded at 0hr and 8hr and audio-recorded throughout. Immediately after the final pseudofeeding recording, birds were fully fed and offered food every 1 hour for the remainder of the day and fed to satiety. The next day, the birds were offered food every 1.5 hours as was customary for hand-reared individuals. Between 1300 - 1500, the birds were again audio- and video-recorded during one such feeding session.

The 0hr, 8hr, and ‘Day After’ videos were then analyzed by an individual blind to the age of the bird, the time-point, and experimental hypotheses. Specifically, we assessed what type of call (A or B) was produced at the first position of each begging bout. A begging bout was defined as the vocalizations produced between pseudofeedings -the syringe being inserted into the oral cavity of the bird which results in the temporary suspension of vocalizations. The percent of calls that were B calls at the first position was calculated for each bird for each time-point. Time-points (0hr, 8hr, ‘Day After’) were then compared using a One-way ANOVA with Tukey’s Multiple Comparison Test.

Results and conclusion

A One-way ANOVA revealed that the percent of B calls produced in the first call position significantly differed between 0hr, 8hr, and ‘Day After’ $F(1, 3) = 6.873$, $p = 0.0116$. Tukey’s Post-hoc tests revealed significant differences

between time-periods and are shown in Figure 4.1. While some P16 birds were already producing B calls before the onset of hunger stress (0hr), the percent of begging bouts that began with a B call increased dramatically from a mean of 33.3% to 80.8% by 8hours of hunger stress. After the birds had been fed and returned to a normal predictable feeding schedule, the birds were assayed again the next day and B call production remained elevated (Figure 4.1, 'Day After').

There are a number of interesting observations to be made from the current data. First, and most obviously, a period of hunger stress can increase the production of B calls in the first call position in a begging bout (0hr versus 8hr). However, this effect is statistically supported even if all the calls produced during food begging are considered (data not shown), showing that hunger increases the production of B calls -regardless of call position. These data demonstrates that begging canary fledglings change the call types produced in response to hunger stress.

Interestingly, after hunger stress, 2 of the birds stopped vocalizing altogether while food begging (note that only four data points are present at 8hr from the original 6 at 0hr). Importantly, the four birds that did produce B calls at 0hr are not the same 4 animals still producing B calls at 8hrs, so that using or not using B calls while begging did not predict which birds would stop vocalizing after hunger stress. The behavior of these two fledglings during a feeding session also changed: they would no longer stay stationary throughout a feeding session but

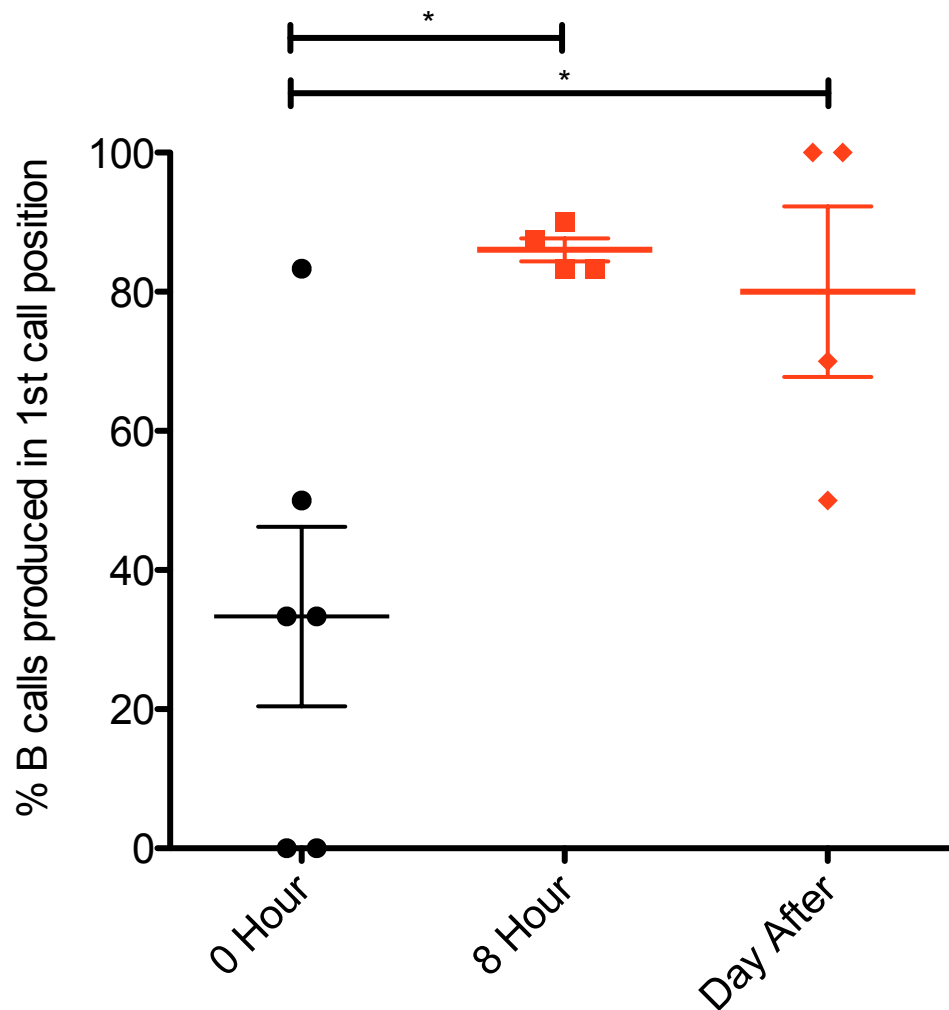


Figure 4.1: In P16 birds, an 8hr bout of hunger stress induces greater B call production and this elevated production remains. Six P16 fledglings experienced 8 hours of hunger stress in the middle of the day. Birds produced some B calls before the onset of hunger stress (**0 Hour**). The percent of calls in the first call position in a begging bout significantly increased after 8 hours of hunger stress, but two birds stopped vocalizing during food begging (**8 Hour**). The animals were then fully fed for the remainder of the experiment. The following day, between 1100 - 1300, B call production was assessed during food begging. The elevated production of B calls remained elevated (**Day After**). All statistically significant comparisons shown. * = $p \leq 0.05$, ** = $p \leq 0.01$.

would continuously back up after every instance of feeding, the head bowed, beak closed, and wings flapping. Intriguingly, the new feeding behavior resembled that of nearly independent fledglings (P24 - P25), suggesting that hunger stress may have caused the acceleration of development towards independence in some animals.

Experiment 2: What are the effects of hunger stress in P14 canaries?

I next asked if a similar bout of hunger stress might speed up the development of some of other P16-associated behaviors (B calls, fledging, lateralized calls). When P14, 6 hand-raised canaries (3 females, 3 males) experienced the same stress and recording paradigm as explained in experiment 1. Time-points (0hr, 8hr, 'Day After') were then compared using a One-way ANOVA with Tukey's Multiple Comparison Test.

Results and conclusion

A One-way ANOVA revealed that the percent of B calls produced in the first call position significantly differed between 0hr, 8hr, and 'Day After' $F(1, 3) = 9.582$, $p = 0.0015$. Tukey's Post-hoc tests revealed significant differences between time-periods and are shown in Figure 4.2.

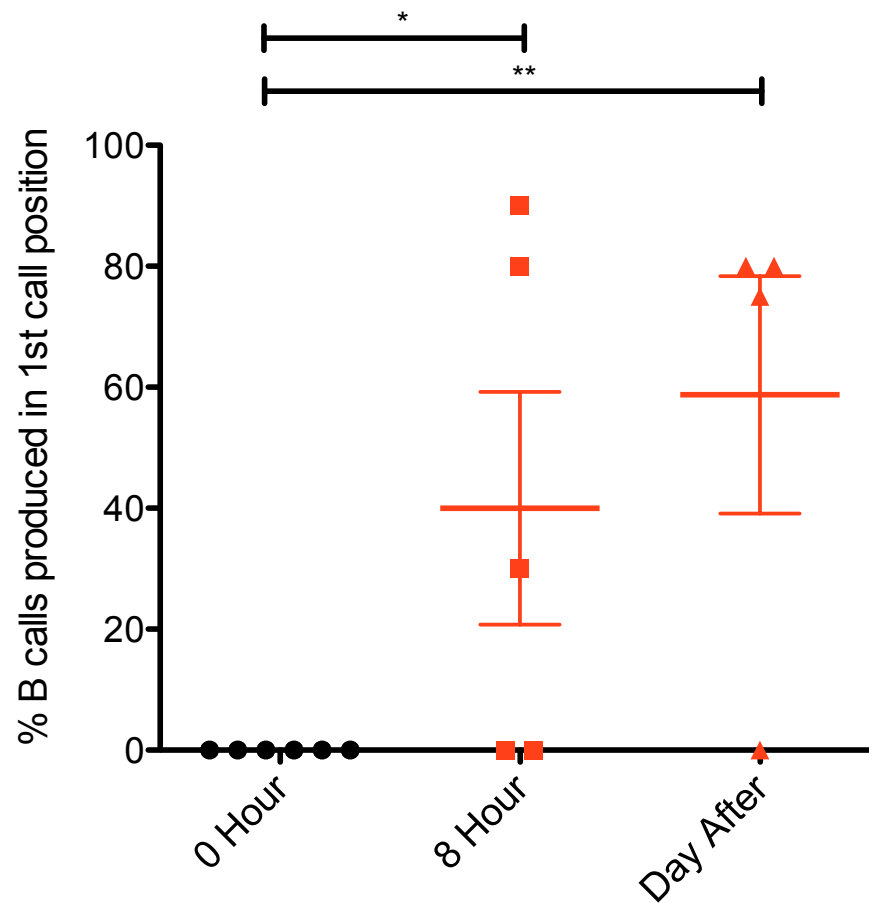


Figure 4.2: In P14 birds, an 8hr bout of hunger stress induces the production of B calls and their production remains. Six P14 nestlings experienced 8 hours of hunger stress in the middle of the day. None of the birds produced B calls during food begging before the onset of hunger stress (**0 Hour**). An 8 hour bout of hunger stress induced the production of B calls in 3 of the individuals. Two birds did not produce B calls and one bird stopped vocalizing during food begging (**8 Hour**). The animals were then fully fed for the remainder of the experiment. The following day, between 1100 - 1300, B call production was assessed during food begging. B calls continued to be produced and one more animal stopped vocalizing during food begging (**Day After**). All statistically significant comparisons shown. * = $p \leq 0.05$, ** = $p \leq 0.01$.

In my hands, P14 canaries do not normally produce B calls (Figures 2.22, 4.2 0hr). However, one bout of hunger stress induced the production of type B begging calls in P14 birds, providing direct evidence for the interpretation that a lack of feeding by parents can result in the accelerated appearance of developmental markers.

However, it is important to note that not all P14 birds produced B calls 8 hours post-hunger stress (Figure 4.2 8hr). Of the six P14 birds tested here, one stopped vocalizing while food begging at the 8hr time-point and another the following day (Figure 4.2), which is very atypical for birds of this age. The behavior of these birds also more closely typified that of older fledglings, with more mobility in the nest, and perching during feeding. These observations suggest that several stereotyped developmental transitions can be shifted earlier by environmental stressors.

Experiment 3: Does hunger stress induce fledging?

I next asked whether fledging behaviors were induced by hunger stress. The six canary hatchlings from the previous experiment and an additional seven hand-reared canaries that experienced the same hunger stress protocol (n = 13 total) were assayed for the onset of fledging. Specifically, the posture of birds while in the nest and during feeding was assessed at 0hr, 8hr, and the following

day. Perching on the rim of the nest cup while at rest before feeding or backing up to perch on the rim of the feeding platform during feeding was scored as fledged (Figure 2.24) by an investigator blind to the hypotheses of the study.

Results and conclusion

No P14 birds were scored as fledged at the 0hr time point either before feeding while the bird was still in the nest or during feeding. However, a period of hunger caused nestlings to begin to fledge sooner (Figure 4.3). All birds that fledged at P14 still showed fledging-like postures the following day such as perching while feeding, indicating that this change could be permanent.

The current data also shows that there is apparent synchrony between the onset of fledging and a vocalization that may help to locate the fledgling -B calls- as their appearance coincides in normally fed birds at P16 or in those shifted by food deprivation stress to P14. Whether similar mechanisms govern their onset or whether B call production lies dormant until needed is unknown. Evolutionarily, their co-expression regardless of fledging timing would appear to be a winning strategy: If when nestlings fledge is developmentally variable, for example based on nutritional allotment, their need to communicate locatability to feeding parents follows along and B calls appear structurally well suited to communicate location.

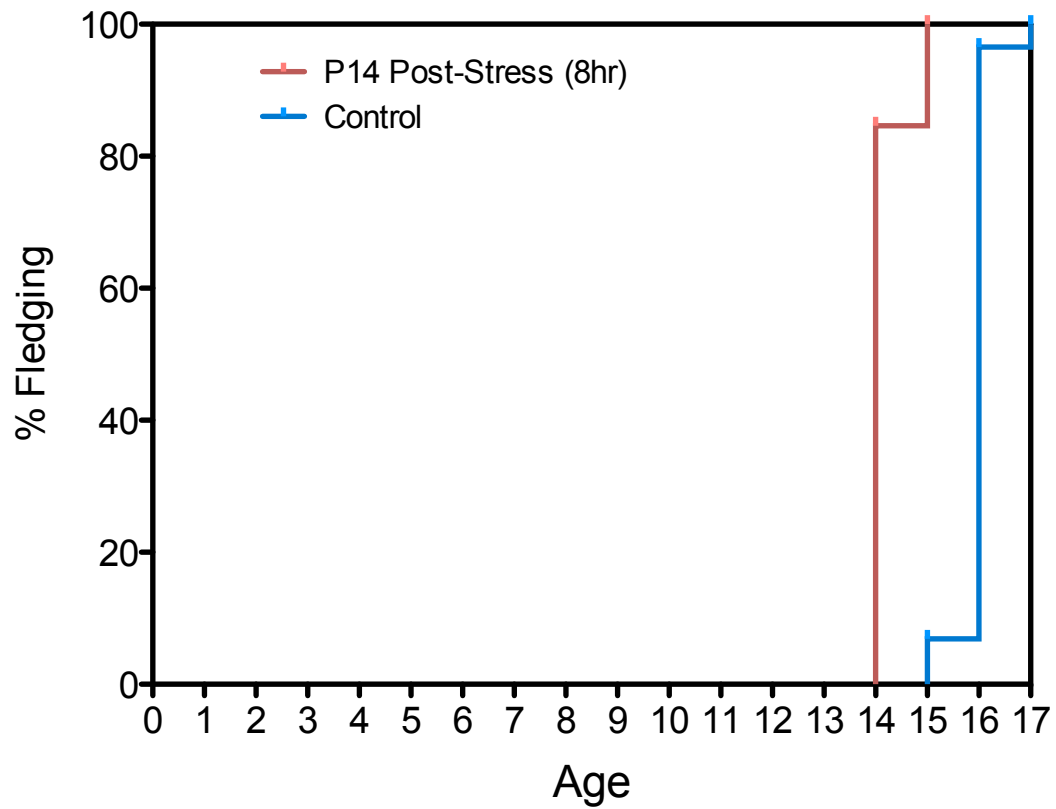


Figure 4.3: An 8hr bout of hunger stress induces fledging in P14 canaries. Under high nutrition conditions (fed constantly), canary nestlings predominantly fledge at P16 (blue line). However, an 8 hour bout of hunger stress induces fledging in P14 canaries (red line).

Experiment 4: Does hunger stress accelerate lateralized food begging calls?

The last P16-associated phenomenon that I wanted to assess was the asymmetric effect of left denervation. To test if left-denervation effects were also shifted earlier following stress, 8 hand-raised P14 canaries (5 females, 3 males) experienced an identical hunger stress protocol as in experiments 1, 2, and 3. The next day (P15), the birds were either left ($n = 6$) or right ($n = 2$) denervated using isoflurane (See chapter 3 for specific methods). Begging calls were recorded before and after denervation (within 2 hours of each other).

Results and conclusions

Syringeal denervations in well-fed P15 individuals did not have destabilizing effects on the structure of the food begging call (Figure 3.6). Following a bout of hunger stress, however, P15 left, but not right, denervated birds showed dramatic changes to the begging call only seen in P16 and older individuals (Figure 4.4).

These results show that hunger stress has specific effects on the lateralized effects of denervation. Left, but not right denervations have strong effects on begging calls, suggesting that the effects of hunger stress at least in

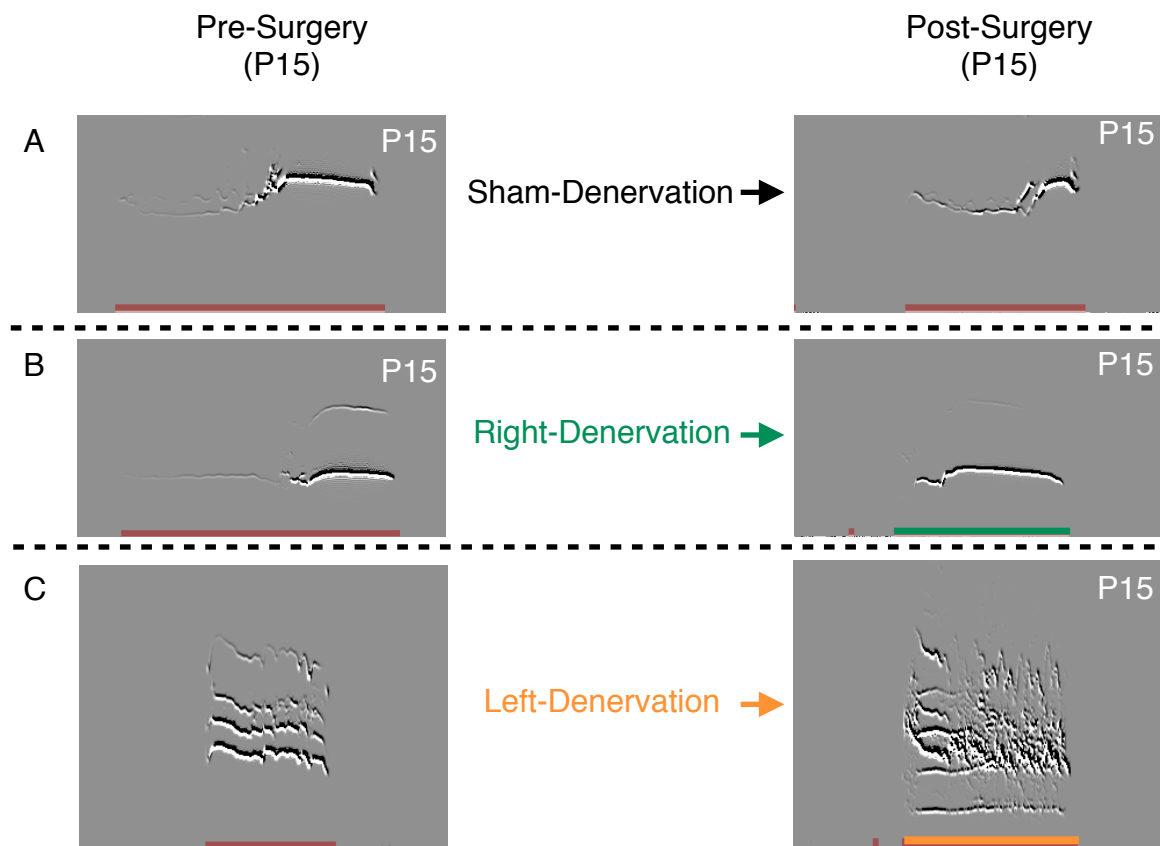


Figure 4.4: Food deprivation induces lateralization of begging call. All birds pictured here experienced a bout of food deprivation stress at P14. Birds underwent denervation or sham surgery at P15 under isoflurane. Sham surgeries (**A**) and Right denervations (**B**) had little effect on the structure of begging calls. Left denervations (**C**) the day after a bout of food deprivation stress, however resulted in noisy vocalizations.

part recapitulated normal developmental events that lead to the asymmetric effects I see in P16 canaries.

Final thoughts

The rapidity of the response to hunger is surprising. Birds of these ages are fed every 2 hours by me and thus 8 hours of no food really translates to an additional 6 hours without food than normal. Yet, because of these 6 extra hours, birds show fledging behavior, begin to produce calls they never otherwise would, and have asymmetric production of begging calls typical of older birds. It suggests that the underlying neural tools necessary to perform these behaviors may be in place but perhaps dormant. The data do not speak to specific mechanisms that cause such changes in behavior, but stress hormones may be a place to begin. Glucocorticoid receptors are found throughout the song system, including nucleus RA and HVC (**Shahbazi, Schmidt, & Carruth, 2011; K. Suzuki, Matsunaga, Kobayashi, & Okanoya, 2011**). Moreover, as early as P10, European starlings (See Appendix 4) show increases in plasma as well as brain corticosterone levels following 45 minutes of restraint stress (**K. L. Schmidt, Chin, Shah, & Soma, 2009**), showing that glucocorticoids in nestlings are stress-responsive.

Bouts of hunger caused B call production but, intriguingly, some of the birds stopped food begging altogether. While my interpretation is that hunger advanced the time towards fledging and thus the stoppage of begging further reflects this, there is an alternative explanation. It is possible that hunger caused canary hatchlings to grow fearful of the feeding parent -namely, me- particularly, as I was no longer providing food and am considerably larger and of deviant morphology from the parents that evolution may have shaped them to recognize. Unfortunately, I did not test for this possibility and future experiments may be necessary to better establish the cause of vocalization loss during food begging in canary hatchlings.

If hungry birds wanted to signal hunger state and A and B calls were equivalent in signaling this nutritional need, we would not have found an increase in the proportion of B calls, just an increase in the total number of calls produced, of both type and B. Perhaps an increased hunger state is accompanied by a greater percentage of B call production and as such serves as an honest signal of need. Yet, again, this is not likely to be the case as elevated levels of B call production remain after almost 20 hours since the end of hunger stress when the birds are being fully fed. If the percentage of B calls produced signaled need honestly, B calls would have been rarer the following day. Instead, it is clear that hungrier birds give more exuberant food begging performances (**Kedar et al., 2000; Lacovides & Evans, 1998; M. L. Leonard & A. G. Horn, 2001; Leonard, Horn, & Parks, 2003**). Perhaps type B vocalizations are the more exuberant of

the two begging call types and have a different value in eliciting parental feeding. This is still somewhat speculative of course as the response to A versus B call production by feeding parents will need to be studied to determine if the calls carry different weight as signals of need.

Lastly, it is intriguing to consider that time to fledge may be advanced by insufficient parental feeding. Perhaps the P16 fledging data from my hand-reared birds (Figure 2.24) showed an artificially tight distribution. Nestlings in the wild undoubtedly experience greater variability in food allotment than the regular 2 hour feedings to satiety that I provided. If this is the case, and it probably is, then the response of the nestlings to not being fed by moving the transition to independence sooner appears like a logical one and perhaps an evolutionarily adaptive strategy. Whether a bird of such young age may be able to survive independently is not known and I have not tested for it. Whether such young birds become independent or their behavior simply dramatically changes after bouts of hunger deprivation is unknown and should be experimentally clarified.

Chapter 5: Closing Thoughts

Vocal ontogeny vs. The 3-stage model of song learning

Songbirds are enjoyed for their beauty and, also, for their song. A scientific field has emerged around the study of adult song as a way to understand the neurobiology of vocal learning and the motor control of complex behavior. Canonically, song has been viewed as having three stages: subsong, plastic song, and crystallized adult song (Nottebohm, 2005; Wilbrecht & Nottebohm, 2003). However, it had been long postulated that this view was perhaps too narrow (F., 1972; Nottebohm, 1970) and should include earlier vocalizations such as begging calls. However, the only evidence to suggest a link was sonographic analysis that showed that some birds incorporated the begging call into their early subsong. Yet, whether these were the same vocalization or ones that looked similar was unknown. Since then, a number of persuasive studies have reinvigorated the argument about when song really begins. Two papers in budgerigars (parakeets) showed that lesions to forebrain nuclei used in adult vocalizations affected the late, but not early begging call of juveniles (Heaton & Brauth, 2000a, 2000b). Moreover, deafening nestlings affected the late begging call, suggesting the use of auditory feedback, normally a feature associated with adult learned vocalizations such as song, in the production of begging calls (Heaton & Brauth, 1999). However, budgerigars stay in the nest for ~2 months, much longer than the vast majority of songbirds (except corvids), which partially

complicates the argument as budgerigars are actively learning adult vocalizations at this time. A study in chipping sparrows added clarification by showing that the begging call of males, but not females, utilized forebrain circuitry and auditory feedback in the very late begging call. These are both features of adult song, supporting the case that the very late begging call may be a “harbinger of song” (Liu et al., 2009).

The studies I present in this thesis add to this body of work by showing that lateralization, also a feature of adult song, is present in begging calls at a stage where only begging calls are being produced. Furthermore, I have found that begging call itself can be subdivided into at least two stages, pre- and post-fledging. Indeed, it is interesting to reflect on the fact that the previously mentioned experiments found that late, but not early begging call ontogeny was affected by central lesions or deafenings and sexual dimorphism in the begging call occurred only in late begging calls (M.E. Hauber, 2003; Saino et al., 2003; Saino et al., 2008). All of these studies showed effects after, but not before fledging, but none mention it. Could the organizing principle behind all of these results be the time of fledging? Either way, I describe complexities in the begging call not appreciated before and that appear associated with the time of fledging.

My data further suggest a succession of stages in begging call development: In the earliest stage (P8 - P9), begging calls result from expired air flowing past the syrinx, without any involvement of the syringeal muscles or their

innervation (Figure 3.2). In the second stage, the syringeal muscles play some role in the begging call, as unilateral denervations can silence a sound source during **A** call production (Figure 3.7), but the majority of the resulting call structure is defined by expiratory pressure (Figure 2.29). In the last stage, the syringeal musculature may play a leading role in modulating the frequency of the sounds produced, a situation that would explain the much greater modulation of frequency in the **B** calls. Thus, our data supports a model of increasing involvement of syringeal musculature used in begging call production across begging ontogeny. By this third stage, the level of syringeal control may already allow for the greater diversity of sounds the bird will produce during subsong, plastic song and adult song, though finer motor control may still be added after the end of the food-begging call stage. Thus, with features such as sexual dimorphism, forebrain nuclei involvement, auditory feedback, lateralization of calls, and syringeal musculature all developing adult-like phenotypes across begging call ontogeny, the case is stronger than ever that nestling begging calls and the onset of song learning may be related.

This idea matters. If we see song as having roots in the very earliest calls produced, then the evolution of vocal learning has a central logic: the modification of preexisting calls through the utilization of increasingly complex neural circuits to achieve complexity. In fact, we see this when the **B** call emerges and we see it again across song learning. This may in fact prove to be a model for understanding how complex behaviors emerge from the modification of

simpler ones. For example, work has already proposed that flight in birds may have emerged from simpler wing movements (Dial, Jackson, & Segre, 2008). Or, more speculative, is a human infant's cry related to the evolution of human language?

The life history of our animals matters

The study of the brain always relates to behavior. Yet, we so often look at our animals as puddles of biology and keep them in small confined spaces, in social isolation, and test them in behavioral paradigms that often make no ecological sense. We have, in a sense, molded the animals we study into the laboratories we build. Yet, simple modifications of assays towards more ecologically valid tests, have yielded fantastic insights (Derdikman et al., 2009; Fyhn, Hafting, Treves, Moser, & Moser, 2007; Hafting, Fyhn, Bonnevie, Moser, & Moser, 2008; Raby, Alexis, Dickinson, & Clayton, 2007; Y. Zhang, Lu, & Bargmann, 2005). An emphasis on the behavior of the animal, and of needing to consider the life history of the bird, were critical to recognizing the special relationship that fledging, an important life-event for songbirds, seems to play with B calls and lateralization.

Fledging, B calls, and lateralization occur together, whether in normally hand-reared birds or in food-deprived birds. While we do not know if this is a persistent coincidence, it is interesting to consider that their co-emergence may

be related. B calls certainly appear structurally well suited for the role of communicating locatability including broad frequency range, frequency modulation, and abrupt onset. Furthermore, as the calls can be used in contact-like call settings, as for example, when the bird is visually isolated, it would make some sense that B calls would be relied on at the time the bird leaves the nest. Furthermore, fledging from the nest presents many new challenges to a bird. Whereas before one was stationary, one is now mobile. Predators had to enter the nest, and one is now in the open. Food came to you and now you may have to significantly move towards it. The correlative nature of the emergence from the nest with the emergence of asymmetric vocal production is interesting and may possibly just hint the role of lateralized behaviors.

The role of asymmetry

Many studies have documented lateralized behaviors in humans and other vertebrates. Yet, why behaviors should be lateralized at all is still essentially guesswork. One theory is that lateralization may allow for the compartmentalization of the complex world: There is a lot to keep track of and compute and centralized processing gives the best hopes of not getting wires crossed and for making rapid decisions. If this is the case, it is interesting that lateralization would emerge in my canaries at the precise time they reach outside of the nest and meet this complex world for themselves. My work certainly does not show enough to conclude these things, but it may suggest future lines of

research in a now better defined model for those that may wish to describe how behaviors lateralize so as to better speak on why they do.

Future directions

I have not carried out the necessary experiments to determine the role of hormones in the stress effects I see, but the food deprivation work that I present does suggest strategies to understand the mechanism that may drive the vast number of behavioral changes at P16. That I can induce P16-related behaviors earlier by a bout of hunger suggests that in this paradigm I may recapitulate the mechanism that normally causes the behavioral changes at P16. If adrenally released glucocorticoids (GCs) play a role, could adrenalectomies or injections of metyrapone, a drug that blocks GC action, before P16 delay the onset of P16-related behaviors? Could injections of GCs at P14 speed up the onset of P16 behaviors and recapitulate my hunger stress studies? If so, what parts of the nestling brain express GC receptors? Do lesions to any of these nuclei affect the onset of P16-related behaviors? The answer is that I do not know but I hope that these are lines of research other studies will address. They may get us closer to understanding what I initially set out to discover -how are behavioral asymmetries in vertebrates established?

An important consideration to all of the work presented herein is that the birds were hand-fed in isolation and thus there was no sib-sib interaction/

competition. As a result, my work may not be completely ecologically valid and experiments should be undertaken to clarify whether my findings hold true in populated nests.

Future experiments should focus on the contribution of DM to begging calls. First, DM projects to n12 in adults, though whether this is the case in juveniles is not known. Moreover, in work not presented herein, DM showed high cytochrome-oxidase signal in P14 - P17 hatchlings, suggesting metabolic maturity not seen in RA or HVC (See Chapter 3).

Lastly, to best understand the nature of the noisy sounds P16 and older canary hatchlings produce that I have tried to clarify herein, unilateral plugging of each bronchus later coupled with unilateral denervations of the Ts nerve need to be completed. Ultimately, these are the finest experiments to establish the role of each syringeal half during begging call production. Rod Suthers and I tried to undertake these experiments but were unsuccessful -it is my hope others will have greater fortune. A clearer understanding of what each half contributes during begging call can then lead to strong hypothesis driven experiments in the central nervous system.

A closing note

I have made many mistakes during my research, made bad decisions, gone down unfruitful avenues for too long and not explored others sufficiently.

Yet, there is one mistake that I have begun to particularly appreciate as a blessing. I knew I was likely the only person working on the ontogenesis of vocal dominance in canaries so I allowed myself a great deal of room to play and explore begging behavior to try and see it with naive eyes in order to acquire new insights. It led to the hunger studies in the 4th chapter, the canary mother discrimination test in the 2nd, the begging bout position analysis, the hand-rearing of zebra-finches until they were well past 100 days of age (not presented), and the hand-raising of 4 wild species of birds just to see begging behavior from another view. However, and this is the mistake I am referring to, I also tried not to read papers on begging calls. I tried, as much as possible, to do my research on an intellectual island, believing reading the work of others before I had started to form some opinions of my own would only bias what I saw. The result was that when I started to read papers I found that many of my discoveries had already been made. While sometimes disheartening, it was also reassuring as I had come to the same conclusions as many other experts, suggesting I was not completely lost. I spent a lot of time doing things others had done and I sometimes wish I would not have been so stubborn about being on my own. However, I also got to make a lot of discoveries. As I am now likely leaving science, perhaps it was a blessing that I got to make so many. May they make up for the years I will not be doing something I have loved for so long.

Appendix 1: General Methods for all Experiments

Software used:

Sound Analysis Pro 2.0 and 2011 (Ofer Tchernichovski)

Excel 2009 (Microsoft)

Numbers 2011 (Apple)

Goldwave (Goldwave Inc.)

Prism V 5.0 (GraphPad Software, 2011)

SPSS V16.0 (IBM Inc.)

Pages 2011 (Apple)

Keynote 2011 (Apple)

iMovie 2009 (Apple)

Igor Pro 5.05 (WaveMetrics)

Subjects:

The care of animals used in these experiments followed the standards set by the American Association of Laboratory Animal Care and the Rockefeller University Animal Use and Care Committee. For chapters 2-4, hatchling canaries of the Waterslager strain were used from the Rockefeller Field Research Station's breeding colony in Millbrook, NY. The date of hatching for each bird is considered P0, the day after P1 and so forth. Hatchling canaries were collected from nests at P5 - P10 and banded around the legs for identification. All birds were moved to a nest-cup and kept with 2 - 4 other canaries of similar age but

not necessarily from the same brood. Hatchlings in these studies come from three breeding rooms that were light-shifted so that at least one canary room was always experiencing 'Spring'-like light/dark cycle. The adult birds within these rooms experienced light/dark shifts once in their life, while the room's light cycle was being shifted, and were then kept therein for the remainder of their lifetime. Adult canaries used in studies herein were collected from a room that had not experienced unnatural light/dark shifts within 24 months of collection. The season of their collection is noted by individual where appropriate.

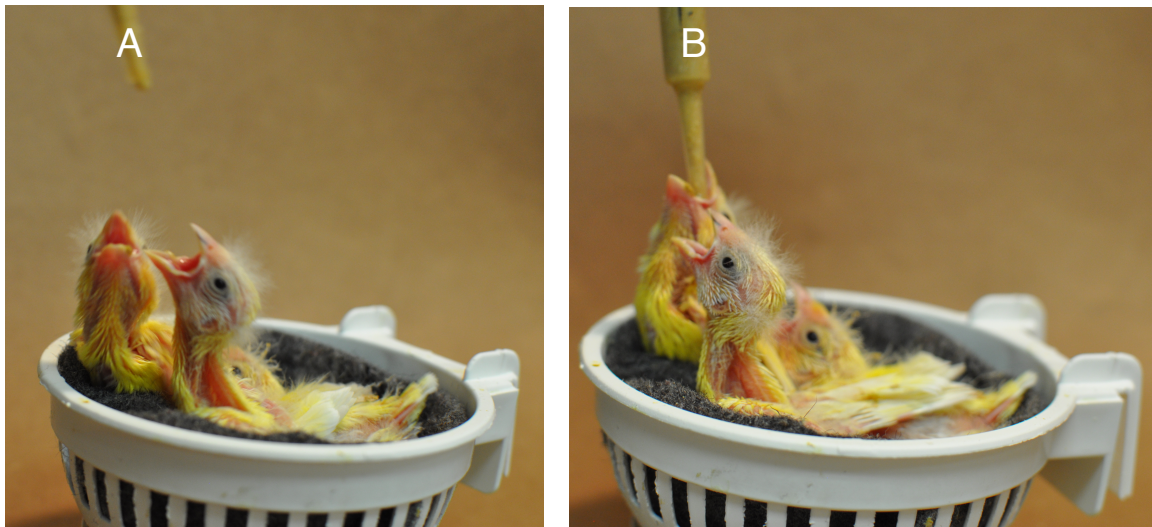
Nestling Feeding:

Nestlings were fed a diet of Avia Vitamins (Nutra-Vet, Millbrook, NY) mixed with hot water until a thick but still runny consistency was reached. Birds were fed through a needleless syringe every 1.5-2 hours from 6am to 9pm daily. To induce begging calls, the syringe tip was placed 2-3 inches from the bird's beak and slowly swayed ~1 inch from left to right. After a number of begging calls were elicited, the syringe was placed in the oral cavity up until the proximal end of the beak and a small amount of food was deposited. The pattern of eliciting calls and feeding was repeated until the birds no longer accepted food (Appendix Figure 1.1).

Perfusion of tissue:

When noted, animals were deeply anesthetized with 1:5 Nembutal solution (See '1:5 Nebutal' in appendix 3). When unresponsive to a toe pinch, birds were

intracardially perfused with 40mls of 0.9% saline solution followed by 40mls of 4% paraformaldehyde (PFA: see '4% PFA recipe' in appendix 3) as a fixative using a pump.



Appendix Figure 1.1: Canary hatchlings being hand fed. Three P12 canaries food begging. **A)** Note the extended neck, beak that is agape and the feeding syringe held nearby but out of reach. **B)** The feeding syringe is brought just posterior to the posterior end of the beak and some food is deposited in the oral cavity. The process is repeated for all birds until food begging stops.

Sectioning of perfused tissue:

The brains were carefully removed and postfixed in 4% PFA for 1 hour, then taken through increasing sucrose concentrations: 5% (2 hours), 15% (overnight), 30% (overnight). Following the 30% sucrose step, brains were placed in a plastic brain mold containing Neg-50 blocking medium (Thermo Scientific, cat. 6502) and covered. Brains were allowed to rest in Neg-50 media for 1 hour and were subsequently frozen using dry ice. Frozen brain blocks were stored at -80 C°. When ready for sectioning, brains were placed in a -20 C° environment for 2 hours and then sectioned coronally unless otherwise stated on a cryostat at a thickness of 40 µms.

Recording:

All hatchling birds were recorded using the 'Live Recording' module within Sound Analysis Pro. A microphone was held within ~1-3 inches of vocalizing hatchling birds during the entire feeding bout.

Recording preparation:

Unlike adult song where an animal may sing a complete song hundreds of times per day with passive recording, begging calls are laborious to attain and sometimes only 50-100 calls per day may be produced or recorded per animal. Therefore, to increase the signal (calls) to noise (background sounds, wing flaps, microphone movements) ratio, all sound files were prepared for analysis in four steps. First, all sound files containing only noise were manually deleted.

Secondly, every remaining sound file was opened in Goldwave and every non-vocalization sound was manually deleted from the .WAV file. Thirdly, using Sound Analysis Pro's 'Batch Analysis' module, every call was analyzed for call characteristics using both amplitude and entropy cut-offs specific to that bird's vocalizations on that day in a manner to best segment sound files into calls versus quiet. The segmentation process, which results in the visual underlining in red of sounds that meet selection criteria, guides the analysis portion of SAP whereby only segments that are underlined are analyzed and each continuous underline is treated as one continuous sound. The resulting call feature analysis was then exported to Excel, and calls shorter than 49 ms or of average call frequencies lower than 2.5 kHz were deleted as experience has shown that these are noise, not food begging calls.

Video Recording:

All recordings were made on an Olympus Stylus 1010, 10.1 megapixel camera mounted on a tripod. Files were moved onto a hard-drive and, if needed, trimmed or given a title in Apple iMovie 2009. All video alterations are noted on a movie by movie basis.

Statistics and Graphing:

All data was collected in Excel 2009 (Microsoft Inc.) and Numbers 2011 (Apple Inc.) and statistical tests performed in SPSS 16.0 (IBM Inc.) or Prism 5.0 (GraphPad Software). Graphing of data was performed in Numbers 2011 or

Prism 5.0. Bar graphs in which data points were particularly variable or did not huddle around the arithmetic mean were changed and represented instead to display individual data points to more accurately represent raw data. In all figures, * = $\leq p$ 0.05, ** = $\leq p$ 0.01, *** = $\leq p$ 0.001.

Appendix 2: Definition of Sound Analysis Terms

Sound Analysis Pro was used to analyze all raw sound files. The definition of the variables referenced in this thesis that resulted from those analyses are found below. The definitions and images used here are slightly modified for ease of reading and appropriateness from those originally produced by Ofer Tchernichovski and are partially replicated here with permission from the author.

Call duration:

This measures the length of the call in milliseconds.

Mean Frequency:

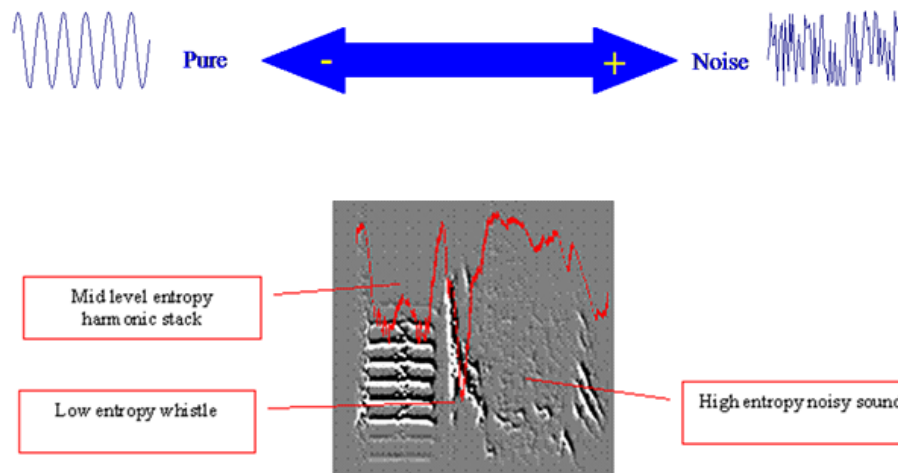
Mean frequency is a pitch measure that assesses the center of the distribution of power across frequencies. Mean frequency provides a smooth estimate of the concentration of spectral power.

Pitch:

The term pitch is used to describe the perceived tone of sounds (high, low, etc). Quantitatively, pitch estimates are measures of the period of oscillation. When the spectral structure is simple, as in a whistle, the pitch can be estimated as the (only) peak in the power spectrum. The location of this peak can be assessed by one of two features: peak frequency, the frequency of highest power, or the mean frequency, the gravity center of the power spectrum.

(Weiner) Entropy

Entropy aims to measure of how ordered a sound is. Specifically, entropy is a measure of the width and uniformity of the power spectrum. Noise is typically broadband with sound energy smeared rather smoothly within the noise range, whereas animal sounds are less uniform in their frequency structure. Wiener entropy is a pure number, that is, it does not have units. On a scale of 0-1, white noise has an entropy value of 1 and complete order, and a pure tone has an



entropy value of 0.

Formal definition: Wiener entropy is a pure number defined as the ratio of geometric mean to arithmetic mean of the spectrum.

Frequency Modulation

Frequency modulation is a measure of how much frequency is changing (or, being 'modulated') across the call. Frequency modulation is estimated based on time and frequency derivatives across frequencies. If the frequency derivatives are much higher than the time derivatives, we say that FM is low and visa versa. Visually, FM is an estimate of the (absolute) slope of frequency traces in reference to the horizontal line.

Appendix 3: Protocols

Included in this appendix are protocols and recipes for various laboratory techniques and chemicals that are mentioned in this thesis. They are included here to more rapidly and effectively communicate protocols and provide a ready resource for anyone that might need them.

NISSL PROTOCOL

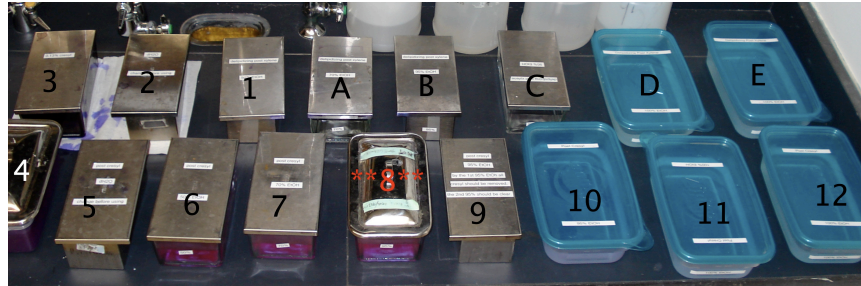
December 17, 2007

Things to be aware of:

For 20um slides:
~1min of Cresyl
Thinner sections require more time.
Thicker shorter.

***Use Acetic Acid to lighten staining if needed. DIP QUICKLY! Do NOT use acetic acid if you have emulsion on your slides!

Change dH2O after every single use.



Nissl Staining Protocol Nottebohm Lab

By Rudy Bellani & Sattie Haripal

- A. 70% Ethanol (2min)
- B. 95% Ethanol (2min)
- C. 95% Ethanol (2min)
- D. 100% Ethanol (2min)
- E. 100% Ethanol (2min)
- Xylene 1 (In hood: 5min)
- Xylene 2 (In hood: 5min)

Delipidizing

- E. 100% Ethanol (2min)
- D. 100 % Ethanol (2min)
- C. 95% Ethanol (2min)
- B. 95% Ethanol (2min)
- A. 70% Ethanol (2min)

Rehydrating

- 1. 50% Ethanol (2min)
- 2. dH2O (30s)
- 3. 0.13% Cresyl (30s-1min)
- 4. dH2O (dip)
- 5. dH2O (30s)
- 6. 50% Ethanol (30s)
- 7. 70% Ethanol (30s)
- 8. ***Acetic Acid*** (dip)
- 9. 95% Ethanol (30s)
- 10. 95% Ethanol (30s)
- 11. 100% Ethanol (30s)
- 12. 100% Ethanol (30s)
- Xylene 1 (In hood: 5 min)
- Xylene 2 (In hood: 10 min)

Staining

Washing

Dehydrating

Coverslip with Krystalon

DNA PURIFICATION

September 2, 2008

Things to be aware of:

For thick or bony tissue, mix tube with digestion buffer a few times during incubation

The digestion procedure can occur for more than overnight, even 2-3 days if needed

For quick and dirty PCR, you can proceed right after digestion at the start of DAY 2



DNA Purification Protocol Nottebohm Lab

By Rudy Bellani

1. Cut piece of tissue and place in sterile 1.5ml eppendorf tube
2. + 600ul Tail Buffer and +3ul of Proteinase K (20mg/ml stock)
3. Incubate at 55C overnight

DAY 1

Digestion

1. Remove from incubation chamber or block and allow to cool down
2. +200 ul 6M ammonium acetate and mix by inversion, do not vortex
3. Place on Ice for 10-15 minutes (increases yield - optional)
4. Place isopropanol and 70% ethanol on ice for future steps
5. Centrifuge at 12000g at 4C for 20 minutes
6. Move supernatant to another tube and spin again at 1200g at 4C for 10 minutes
7. Again move supernatant to a new tube

DAY 2

Removal of undigested tissue and proteins

1. Add 400ul of cold isopropanol and mix by inversion, do not vortex
2. Spin at 12000g at 4C for 15 minutes
3. Discard supernatant, you should see DNA pellet
4. Add 500-600ul of cold 70% ethanol and mix by inversion, do not vortex
5. Spin at 12000g at 4C for 10 minutes
6. Discard Supernatant, place tubes upside down to evaporate for 20-30 minutes
7. Resuspend in 50-100ul of H₂O or TE buffer

Cleaning the DNA

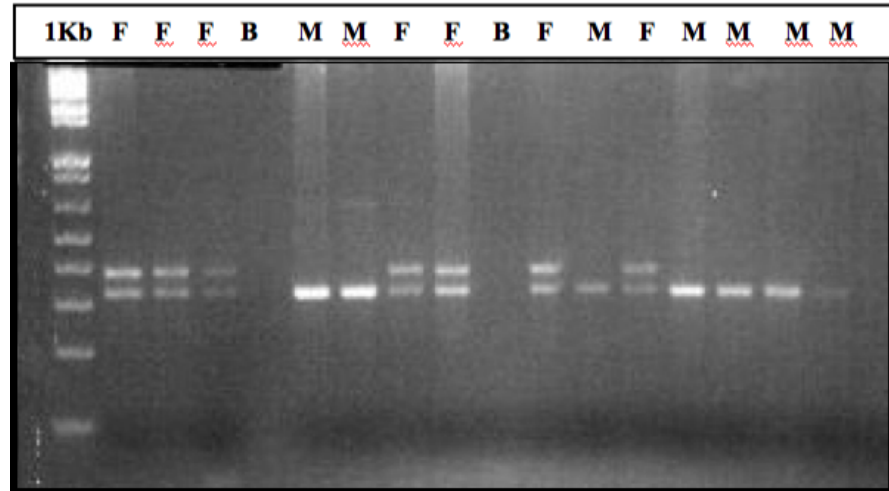
SEXINGPCR

August 15, 2007

Things to be aware of:

This PCR reaction works very robustly and can be started using feather tip, toe clipping, or any other tissue collected post-mortem.

Citation:
Griffiths R, Double MC, Orr K, Dawson RJ. A DNA test to sex most birds. Mol Ecol 1998;7:1071-5.



Bird Sexing PCR Nottebohm Lab

Example of Results:

F = female, M = male, B = blank

By Rudy Bellani, adapted from Griffiths et al (1998), see left panel for full citation.

For each 25ul reaction:

10X Buffer	2.5ul
MgCL2	1ul
DNTP	0.5ul (10mM)
P2 primer	0.5ul
P8 primer	0.5ul
Template	1ul (at least 50ng of DNA)
H2O	19.5 ul (until 25ul taking into account Taq)
Taq	0.5ul

PCR Program:

1. 95C⁰ for 2 minutes
2. 95C⁰ for 20 seconds
3. 52C⁰ for 25 seconds
4. 72C⁰ for 1 minute
5. Go to 2, cycle 35 times
6. Hold at 4C⁰ forever

1:5 NEMBUTAL

April 4, 2009

Things to be aware of:

Make sure to note how much Nembutal was removed from the Nembutal bottle.

Nembutal is a controlled substance and should be handled with care.

Every use of Nembutal solution should be noted.

Ensure that the bottle is stored in a safe place behind two locks.



1:5 Nembutal Solution Nottebohm Lab

By Rudy Bellani, Sattie Haripal

For 10mls total, mix:

4mls dH₂O

0.8ml 100% ethanol

3.2ml propylene glycol (50%)

2ml Nembutal

Prominently write date on bottle.

November 9, 2009

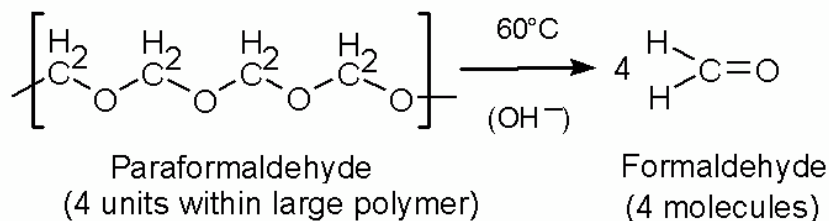
4% PFA RECIPE

Things to be aware of:

NEVER allow PFA mixture to get above 60°C (See Figure)

PFA is best fresh, but can be used for a few days

Filtering is particularly important for perfusions



4% Paraformaldehyde Nottebohm Lab

By Rudy Bellani

1. To 200ml of dH₂O, add 20g of PFA
2. Heat to 58°C on a hot plate with rapid mixing
3. When 58°C, add 3 drop of NaOH immediately
4. Take off of heat, keep mixing, it should turn clear
5. Meanwhile, mix: 200mls sodium phosphate dibasic and 50mls sodium phosphate monobasic
6. Add 250ml mixture (di+mono) once solution is clear and wait until fully dissolved
7. Filter (with the yellow filters using the vacuum)

CYTOCHROME C

July 7, 2010

Things to be aware of:

Songbird Nuclei stand out a great deal with this stain.

Reference:

Rosado, R., Espino, G.G., Rosenfield, D.B., & Helekar, S.A., (2001)

Experience-Dependent changes in cytochrome oxidase staining patterns in zebra finch song nuclei. Society for Neuroscience Abstracts, 27



Cytochrome C Oxidase Staining Nottebohm Lab

By Rudy Bellani

1. Perfuse animal in 4% PFA in PBS
2. Post-fix 24 - 48hr in 4% PFA in PBS
3. Cut at 30 - 200um
4. If cut on freezing sliding microtome, place in 30% sucrose
5. If cut on a vibratome, collect in PBS and store at 4C

DAY 1
Perfusion

DAY 2
Tissue Sectioning

6. Incubate in fresh 0.3% cytochrome C until happy with level of staining (recipe below)
7. Rinse sections 2 times in PBS
8. Mount sections using 0.3% gelatin
9. Dry sections well
10. 50% ethanol: 2.5min (30um) - 5 minutes (200um)
11. 70% ethanol: 2.5min (30um) - 5 minutes (200um)
12. 100% ethanol: 2.5min (30um) - 5 minutes (200um)
13. 100% ethanol: 4min (30um) - 8 minutes (200um)
14. Xylene (15min)
15. Coverslip with DPX

DAY 2 or 3
Cytochrome C stain

Cytochrome C:

0.3% cytochrome C (sigma C-2506)

0.075% DAB (Sigma D-9015)

4% Sucrose

In 0.1 PBS, pH 7.4

For every 1ml of Cytochrome C:

.04g Sucrose

.0003g Cytochrome C (stored at -20C)

.00075g DAB (stored at -20C)

Make recipe for 1ml more than needed to account for pipetting errors. Add sucrose, then cytochrome C and finally DAB. Fill to desired volume with PBS. Mix well. Use immediately.

Appendix 4: Bird Species Referenced



Chaffinch

Fringilla coelebs



White-Crowned Sparrow

Zonotrichia leucophrys



European Starling

Sturnus vulgaris



**Domestic Canary,
Waterslager Strain**

Serinus canaria domestica



Chipping Sparrow

Spizzella passerina



Barn Swallow

Hirundo rustica



Northern Cardinal

Cardinalis cardinalis



Brown Thrasher

Toxostoma rufum



American Redstart

Setophaga ruticilla



Java Sparrow

Padda oryzivora



Grey Catbird

Dumetella carolinensis



Budgerigar (Parakeet)

Melopsittacus undulatus

Photo credits

All photographs not attributed to Rudy Bellani are used with permission from owners or sources or under a creative common license where appropriate.

Chaffinch: <http://www.wildaboutbritain.co.uk/pictures/showphoto.php/photo/62196>

White-Crowned Sparrow: <http://en.wikipedia.org/wiki/File:White-crowned-Sparrow.jpg>

European Starling: Ewe Otter, 2011, <http://eweotter.wordpress.com>

Domestic Canary, Waterslager Strain: Rudy Bellani, 2011

Chipping Sparrow: http://en.wikipedia.org/wiki/File:Spizella-passerina-015_edit.jpg

Barn Swallow: <http://en.wikipedia.org/wiki/File:Landsvale.jpg>

Northern Cardinal: <http://en.wikipedia.org/wiki/File:Cardinal.jpg>

Brown Thrasher: Ken Christison, 2011: <http://burntbridgesbirding.blogspot.com/2011/01/brown-thrasher.html>

American Redstart: http://en.wikipedia.org/wiki/File:American_Redstart_of_Quintana_Texas1.jpg

Java Sparrow: http://en.wikipedia.org/wiki/File:Padda_oryzivora_-_University_of_Hawaii_at_Manoa_campus,_Honolulu,_Hawaii,_USA-8.jpg

Grey Catbird: http://en.wikipedia.org/wiki/File:Dumetella_carolinensis_-_Brendan_T._Byrne_State_Forest,_New_Jersey,_USA-8.jpg

Budgerigar (Parakeet): Mark Coran: http://upload.wikimedia.org/wikipedia/commons/5/56/Rose-ringed_Parakeet_RWD.jpg

Appendix 5: Image Processing

Some images have been altered slightly to better show underlying features. All manipulations were done in iPhoto 2009. The reason for changes and all changes made are listed below.

Figure 2.1 (P9): The underlying Type **A** food-begging call was too quiet to show up clearly when printed and the image was altered to bring out the call. Exposure (0.33), Contrast (100), Saturation (100), Definition (100), Shadows (100), Sharpness (50).

Figure 2.31(P16): The beginning of the **B** call was too quiet to show up clearly when printed and the image was altered to bring out the call. Contrast (100), Saturation (100), Definition (100), Shadows (100).

Figure 3.3 (Sham Denervation): The beginning of the **A** call was too quiet and did not appear clearly and so the image was altered to bring out the call. Exposure (0.04), Contrast (100), Definition (100), Shadows (100), Sharpness (100). The same processing was done on the post-surgery call.

Figure 3.3 (Left Denervation): The call was too quiet and did not appear clearly and so the image was altered to bring out the call. Contrast (100), Definition (100), Highlights (100), Sharpness (100). The same processing was done on the post-surgery call.

“I don’t *work* at all; I only do the things I like to do!”

Detlev Bronk

Appendix 6

In Vivo Electroporation in the Songbird Brain

Songbirds have long been a model for the study of vocal learning (Gardner, Naef, & Nottebohm, 2005; Liu & Nottebohm, 2007; Nottebohm, 1970; Nottebohm & Liu, 2010a; Wilbrecht & Nottebohm, 2003), motor and auditory system function (Fee, 2010; Gentner & Margoliash, 2003; Hahnloser et al., 2002; Keller & Hahnloser, 2009; Leonardo & Fee, 2005; Leonardo & Konishi, 1999; Long & Fee, 2008; Long et al., 2010; Prather, Peters, Nowicki, & Mooney, 2008), neurogenesis (Alvarez-Buylla & Nottebohm, 1988; Goldman & Nottebohm, 1983; Paton & Nottebohm, 1984; Rasika, Alvarez-Buylla, & Nottebohm, 1999), sexual dimorphism (Agate et al., 2003; Arnold, 1997; Arnold & Saltiel, 1979; Nottebohm & Arnold, 1976, 1979), brain asymmetry (Cynx et al., 1992; Goller & Suthers, 1995; Liedvogel et al., 2007; Nottebohm, 1971; Nottebohm et al., 1976; Poirier et al., 2009; Suthers, 1990; Williams et al., 1992; Wiltschko et al., 2002), and magnetoreception (Wiltschko et al., 2002; Zapka et al., 2009). Yet, while behavioral, electrophysiological and histological approaches have led to deep insights in these areas of study, molecular manipulation has not yet gained wide traction across the field. One reason for this is that there are currently no described methods for manipulating gene expression in songbirds without the use of viruses (Agate, Scott, Haripal, Lois, & Nottebohm, 2009; Haesler et al., 2007; Schulz, Haesler, Scharff, & Rochefort, 2010; Scott & Lois, 2007) which is problematic as virus production is expensive, time consuming, requires special

facilities and is therefore a large investment for investigators. Even if such viral-based techniques are adopted, viruses have numerous shortcomings such as limitations on the size of constructs they can vector and generally low infection rates. Moreover, while we have recently described a method for creating transgenic songbirds using viral vectors (Agate et al., 2009), the lack of brain-area specific promoters necessitates alternative tools to manipulate specific brain regions. Thus, in the current set of studies, I undertook the development of a rapid, relatively simple and widely accessible technique to drive gene expression of one or more plasmids in the songbird brain across age, sex, and species and thereby develop a tool for the whole songbird field.

Electroporation is a widely used technique in neuroscience, traditionally in *in ovo* and *in utero* studies, but has recently begun gaining traction as a tool for postnatal *in vivo* work, particularly in rats (Aspalter et al., 2009; Boutin, Diestel, Desoeuvre, Tiveron, & Cremer, 2008; M. Zhang et al., 2009) and mice (Barnabe-Heider et al., 2008; Saito, 2006). Electroporation-mediated gene transfer works predominantly by a combination of cell-permeabilization and electrophoresis, whereby application of electrical pulses across cells or tissues causes brief micropores to appear in cellular membranes (De Vry et al., 2010; Golzio, Teissie, & Rols, 2002; Satkauskas et al., 2005). Thus, when DNA is injected into the tissue of interest, the brief pulses of electricity given across this tissue cause the negatively charged DNA to move towards the positive electrode and enter cells through the created micropores. As electroporation has potential for wide

adoption due to the relative ease of the technique and low entry cost, we sought to develop electroporation for *in vivo* studies, paying particular attention to the diversity of needs in the songbird community.

Materials and Methods:

Experimental Subjects: Zebra finches (*Taeniopygia guttata*), canaries (*Serinus canaria*), and Eastern phoebes (*Sayornis phoebe*) were used in the present study. All adult animals had *ad libitum* access to food and water and were housed in standard group (zebra finches), breeding (canary), or small flight (phoebe) cages. Phoebes were hand reared using modified Lanyon meat mix formula (Lanyon, 1979) and supplemented with wax worms and mealworms. Zebra finches were on a 12:12 L:D cycle and canary and phoebes were on a 14:10 L:D cycle at time of surgeries.

Plasmids and preparation: pCAGGS-EGFP and pCAGGS-DsRed were kindly provided by Yoshiko Takahashi from the Nara Institute of Science and Technology. pCAGGS is a strong constitutive promoter capable of expressing in all cells. DNA was purified using a Qiagen maxi-prep kit (Qiagen cat. 12163), eluted in elution buffer and working solutions of plasmids were 1.5-2.5µg/µl concentrations for all experiments.

Electroporation: An electroporator (BTX) and paddle-like tweezerrode

electrodes (BTX Tweezertrodes cat. 520) were used in all experiments with electroconductive gel (Lectron II Conductivity Gel) applied to exposed skin to maximize electrical transfer into tissue. Due to the wide range of ages of birds used in these studies, and thus differences in cranial tissue makeup, different levels of voltage were used (Table 1).

	Optimal Age	Survival Rate	Voltage Used	Pulses	Pulse Length	Interpulse Length	Success Rate
Hatchling	<i>Canary:</i> P0.5 <i>ZF:</i> P3.5	94%	75V	3	50ms	950ms	100% (n = 12)
Adult	N.A.	100%	150V	3	50ms	950ms	60%* (n = 10)

Table 1: Optimal electroporation settings. Success rate is defined by the percent of birds electroporated within that category that were identified as positively expressing plasmid. *Adult birds show greater variability in amount of transfected cells, with some showing robust expression and others only having a handful of labeled cells.

Experiment 1: Canary hatchlings ($n = 34$). Newly hatched canaries (P0.5) were collected from nests, head-feathers trimmed, and 1-1.5 μ ls of plasmid injected only into the left hemisphere through the soft skin and cranium using a pulled pipette. We allowed 1.5 minutes of rest post-injection and then electroporated with 3 square pulses of 50-125 V for 50ms duration at 950ms intervals. Orientation of the tweezerrode electrodes (Figure 1B) alternated between pulses (Figure 1C). For the purposes of our experiments, 1-3 injections were made per animal. Animals were then immediately returned to their home nest and collected 5 days later. For analysis, animals were euthanized, brains collected, sectioned at 100-200 μ ms on a vibratome and imaged immediately.

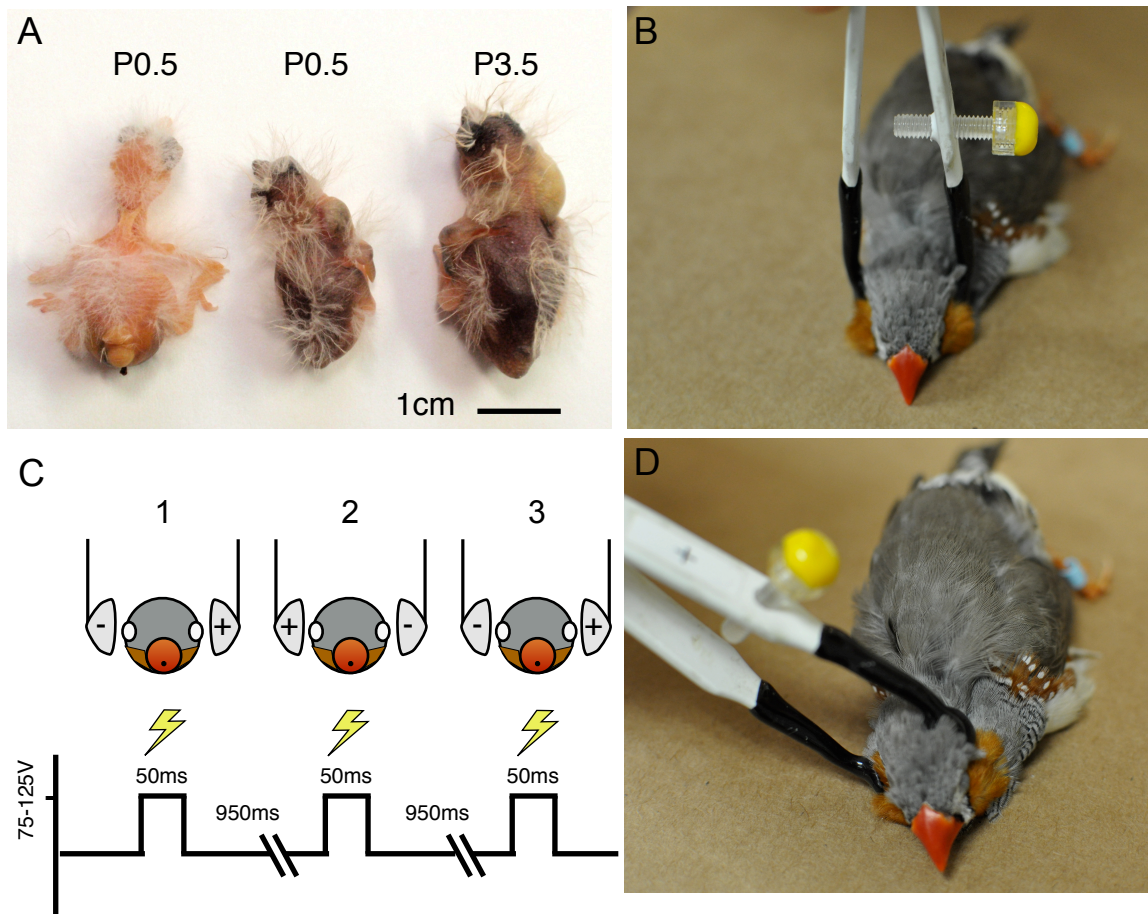


Figure 1: Electroporation in songbirds. **A)** From left to right: A P0.5 canary, a P0.5 zebra finch, and a P3.5 zebra finch. Note that zebra finches have semi-full food pouches and thus may appear larger than they are. **B)** Zebra finch, with electrodes orientated across the brain. **C)** Electroporation schematic. Birds experience 3 square pulses of 75-125V for 50ms with 950ms interpulse intervals. The orientation of electrodes was altered between pulses. **D)** Electroporation of an adult zebra finch as described in Experiment 7.

Experiment 2: Adult Canaries ($n = 8$ Spring-time adult males). To test if electroporation-mediated gene transfer could work in adults, adult canaries were anesthetized using 1:5 Nembutal solution (see Protocols section), a window opened through the skull and stereotaxically injected with 1 μ l of high concentration plasmid (2.5 μ g/ μ l) into the ventricle above HVC using previously published stereotaxic coordinates (Scott & Lois, 2007) with minor modifications, infused over 10 minutes. Head feathers surrounding the ear were removed and animals received 3 square pulses at 150 V for 50ms at 950ms intervals across the scalp (Figure 1C). Birds were allowed to recover under a heat lamp and afterwards returned to their home cage. Five days later, adults were anesthetized, perfused with 4% paraformaldehyde (PFA: See Protocols section), and brains sectioned at 100 μ m on a vibratome and immediately imaged.

Experiment 3: Zebra finch Hatchlings ($n = 21$). To test if *in vivo* electroporation protocols developed in canaries could successfully be utilized in another common lab species, newly hatched zebra finches (P3.5) were treated as in experiment 1, but were all treated with 75 V pulses.

Experiment 4: Adult Zebra finches ($n = 10$ adult males). Adult zebra finches were treated and electroporated as in experiment 2 (Figure 5B) using previously published stereotaxic coordinates (Scott & Lois, 2007).

Experiment 5: Survival Study ($n = 4$ zebra finches). In order to test whether plasmids into hatchlings could express into adulthood, zebra finch hatchlings were electroporated with pCAGGS-DsRed as described in experiment 3, returned to the nest and allowed to sexually mature. At P110-P111, birds were perfused with 4% PFA, post-fixed for 2hrs, taken through increasing concentrations of sucrose solutions over two days, blocked in Neg-50 (Thermo Scientific cat. 6502) and frozen. Brains were then cut at 40 μ m on a cryostat and imaged using confocal microscopy ([info](#)).

Experiment 6: Co-electroporation of plasmids ($n = 3$ canary hatchlings). To test if we could successfully introduce multiple plasmids into cells and thus multiply the experimental potential of electroporation, birds were treated as in experiment 1 except that two plasmids (pCAAGS-DsRed and pCAAGS-EGFP) were mixed at 1:1 DNA concentration (final concentration of each plasmid at 1.25 μ g/ μ l) and injected together. Images of both were collected in serial frontal sections and analyzed for co-expression. For analysis of co-expression, the percentage of large puncta, presumably cell bodies, expressing both GFP and DsRed were calculated from 2 serial sections from 3 zebra finches from experiment 3.

Experiment 7: Electrode orientation ($n = 2$ adult zebra finch males). To test whether altering the orientation of electrodes would allow us to manipulate the location of transgene expression post plasmid injection, I sought to guide gene

expression to the bird hippocampus, a structure dorsal to the ventricle. Birds were treated as in experiment 4, but had the positive electrode placed dorsolateral to the left hemisphere for the duration of the three pulses (Figure 1D).

Experiment 8: Effect on song ($n = 6$ adult male zebra finches). To test if running strong current across the brain had an effect on song, adult male zebra finches (average age = 421 days) were recorded for 1 week in sound isolated chambers using Sound Analysis Pro (SAP) software. Animals were then anesthetized using 1:5 Nembutal, electroporated as in experiment 4 and returned to their recording chamber for another week of recordings. For each bird, 6 randomly selected pre-surgery songs were chosen and analyzed against each other for *Song Similarity* and *% Similarity scores* in Sound Analysis Pro (SAP) by a researcher blind to the treatment, to get a baseline for how similar adult songs are rendition to rendition. Then, the same 6 pre-surgery songs and 6 randomly selected post-surgery songs were compared to each other to determine possible changes due to electroporation.

Experiment 9: Effect on general behavior ($n = 14$ Spring-time female canaries). We further sought to determine the effect of electroporation on other behaviors and thus seven canaries were electroporated as in Experiment 2 but were not collected afterwards (Electroporated Group). A different seven females were treated as in experiment 2 but no current was used (Control Group). All

birds recovered under a heat lamp and were returned to their home cage. The following day, individuals were paired with untreated male adults and allowed to breed. Average number of eggs laid, eggs hatched, hatchlings successfully reared to P7, and hatchlings transferred to another nest due to poor health were recorded for the first clutch laid following electroporation and the two groups, control and those electroporated, compared.

Experiment 10: Eastern Phoebes Hatchlings (n = 3). P3.5-P4.5 Eastern

Phoebes were removed from nests located within Rockefeller University's Field Research Center in Millbrook New York, electroporated as in Experiment 3, and hand-raised until juveniles (~P28.5). Individuals were anesthetized, perfused with 4% PFA, brains collected and imaged as in Experiment 5.

Results:

Strong expression of electroporated constructs in canary and zebra finch hatchlings

To first determine the feasibility of electroporation-mediated gene transfer in songbirds, I began work on canary hatchlings due to the rapidity of the surgeries (see Experiment 1 methods). Various voltages were tested to determine best electroporation practices while assessing canary hatchling health, tissue damage and transgene expression (Table 2). Higher voltages than 75 V resulted in increasing tissue damage and reduced responsitivity post-surgery by hatchlings.

At 75 V, hatchlings appeared unaffected by electroporation and brain tissue remained perceivably healthy, containing no blood clots or lesions (Figure 2A). Importantly, electroporation at 75 V resulted in large swaths of transgene expression at injection sites (Figure 2B, 2D). Using direct injections of plasmid into neural tissue and running current across the scalp resulted in expression predominantly in cells adjacent to the ventricle and occasionally on the dorsal surface of the brain (Figure 2E). No cells were found to express transgene in deep nuclei. All 12 canary hatchlings electroporated at 75 V had DsRed positive swaths of cells (Table 1).

Zebra finch hatchlings are significantly smaller than age-matched canary hatchlings (Figure 1A) and were more sensitive to the electroporation procedures (Table 3). Thus, variously aged zebra finch hatchlings were electroporated at 75 V and animal recovery and surgery success details recorded (Table 3). Zebra finches P2.5 and younger appeared anesthetized post electroporation and post-mortem dissections revealed the presence of small lesions or blood clots on the dorsal surface of the brain. P3.5 and older zebra finches were mildly, if at all, affected by surgical procedures, even eliciting food-begging behaviors (turned head, tongue wagging) immediately post-electroporation. Additionally, areas of DsRed labeled cells consistently became more localized as the age at electroporation increased. Note, for example, the diffusion of transgene expressing cells from a single injection in P3.5 (Figure 2B) versus P5.5 (Figure 2D) zebra finch hatchlings.

Voltage	n	Mortality Rate (%)	Hatchling Behavior post-electroporation	Post-Collection Analysis	Electroporation Success Rate (DsRed+)
50 V	8	0	Strong and active	DsRed+ areas were generally small and spotty	75%
75 V	12	0	Strong and active	DsRed+ areas were generally large, encompassing large swaths of ventricle.	100%
100 V	6	16.7	Hatchlings appeared mildly sedated but breathing rate was strong.	DsRed+ areas were large, but the telencephalon occasionally contained small lesions and a few blood clots, generally in the dorsal telencephalon.	100%
125 V	5	40.0	Hatchlings appeared mildly sedated, but breathing rate was strong. Occasionally mild skin burns on scalp were observed.	DsRed+ areas were small and were accompanied by many observable blood clots and lesions, particularly in the dorsal telencephalon and optic tectum.	40%

Table 2: The effects of varying voltage on electroporation success in hatchling canaries. To determine optimal voltage to drive gene expression, numerous voltages were tested from 50-125 V. Voltages from 50-75 V appeared to have minimal effect on hatchlings, as their behavior was indistinguishable from unelectroporated hatchlings. Birds experiencing these voltages moved vigorously, responded to sound and movement, and even begged for food. While 50 V did not produce robust gene expression, 75 V gave as strong expression as the authors saw with any voltage. Increasing electroporation voltage above 75 resulted in worsening lesions and elevated mortality and decreased reactivity by hatchlings.

Age at Electroporation	n	Mortality Rate (%)	Hatchling Behavior Post-electroporation	Post-Collection Analysis
P0.5	6	50	Weak and appeared mildly sedated.	DsRed+ areas were small and spotty. Great variability between 2 surviving hatchlings. Small lesions were visible on telencephalon.
P1.5	3	66.7	Weak and appeared mildly sedated.	DsRed+ areas were generally diffuse but encompassed large swaths of ventricle. Small blood clots were visible on surface of telencephalon.
P2.5	4	25	Generally strong and active	DsRed+ areas were numerous and encompassed large swaths of ventricle. No lesions or blood clots present in any sample.
P3.5	7	0	Strong and active	DsRed+ areas were numerous and generally encompassed large swaths of ventricle. No lesions or blood clots present in any sample.
P5.5	4	0	Strong and active	DsRed+ areas were dense but less diffuse. No lesions or blood clots present in any sample.

Table 3: Effects of Age on electroporation results in zebra finch

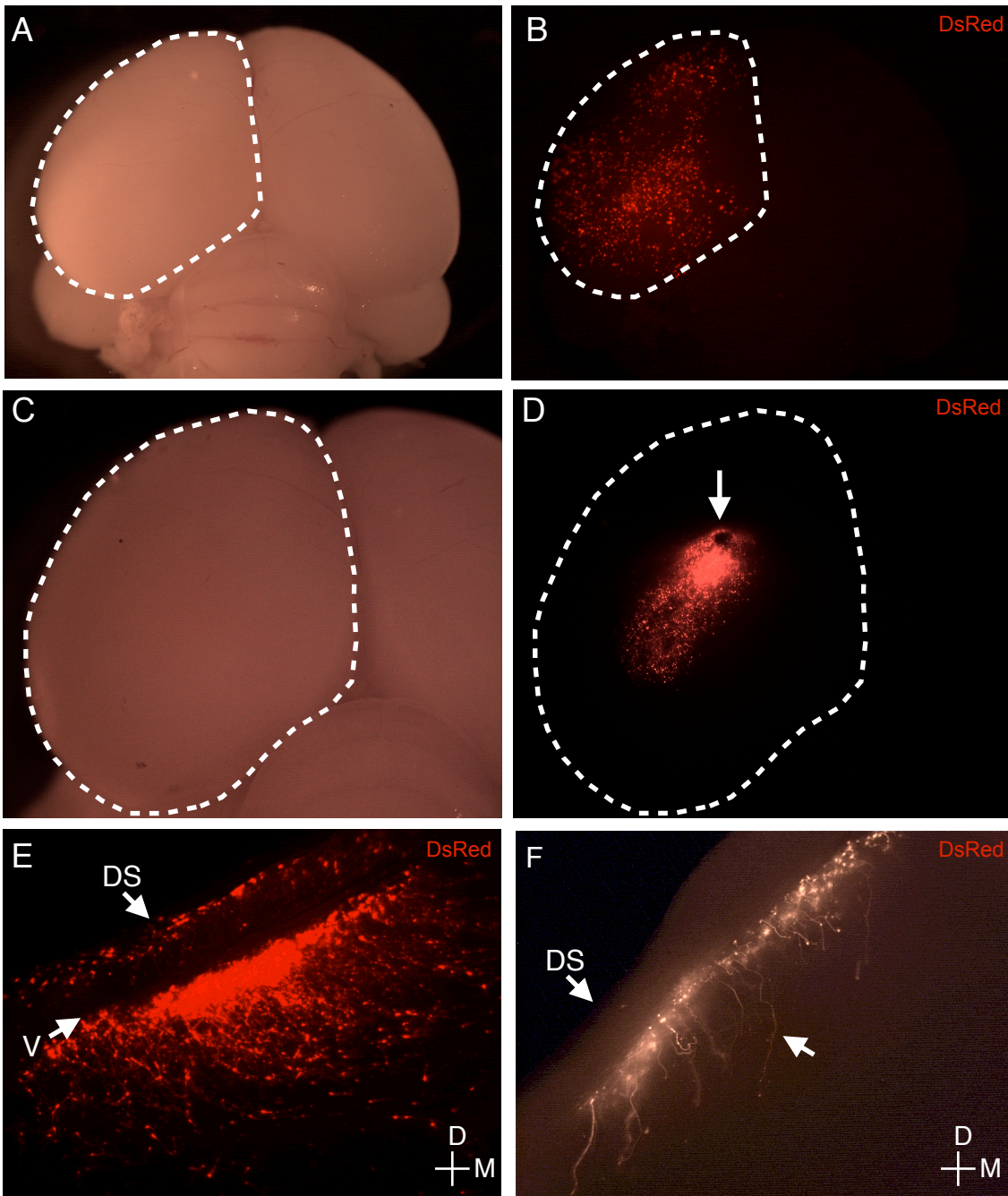
hatchlings. To determine the optimal age to drive gene expression via electroporation in zebra finch hatchlings, variously aged hatchlings were tested at 75 V. While all surviving hatchlings collected 5 days post electroporation had DsRed positive cells in the brain, P0.5 and P1.5 finch hatchlings had higher mortality rates and appeared weaker than P2.5 and older hatchlings. P3.5, like P5.5, finch hatchlings appeared the healthiest and food begged post-electroporation. Electroporating P3.5 hatchlings resulted in generally more widely diffused DsRed positive cells (Figure 1B) while P5.5 electroporations had less diffuse, more densely packed DsRed expressing cells (Figure 1D).

Successful expression of transgenes in canary and zebra finch adults

Adult canary and zebra finch males stereotactically received a single 1 μ l injection of high-concentration (2.5 μ g/ μ l) pCAGGS-DsRed plasmid directly into the ventricle overlying HVC. Adults were electroporated and collected 5 days post surgery (See methods for details). Analysis of adult birds revealed successful and localized transgene expression in the ventricular zone (Figure 2F). Adults, however, had more variable results than hatchlings, with a 64% success rate and greater variance in the amount of DsRed⁺ cells (Table 1). Additionally, adult canaries and zebra finches had even more localized (<1mm diameter) medial-lateral spread of transgene expressing cells in the ventricle than hatchlings, continuing the pattern of greater transgene localization as animals age.

There are also a number of variations to electroporation protocols that may be of use to investigators. First, the co-electroporation of pCAGGS-EGFP and pCAGGS-DsRed plasmids in a 1:1 dilution resulted in high co-expression in cells (>99%, Figure 3). Secondly, manipulating the angle of electrodes such that the positive electrode was dorsal to the left third ventricle (Figure 1D) resulted in transgene expression in the hippocampus (Figure 4B). Lastly, electroporation-mediated transgene expression is long lasting. For example, zebra finch hatchlings receiving plasmid injections into the ventricular zone at P3.5 had robust gene expression at P111, with fully morphologically mature neurons found throughout the brain (Figure 4A),

Figure 2: Robust gene expression via electroporation in canaries and zebra finches. A) Light microscopy image of a P8.5 zebra finch hatchling that received a single pCAGGS-DsRed plasmid injection in the left hemisphere (traced) at P3.5. **B)** Fluorescent microscopy of the same brain as in panel A reveals robust labeling throughout the hemisphere. **C)** Light microscopy image of a P10.5 zebra finch hatchling that received a single pCAGGS-DsRed plasmid injection in the left hemisphere (traced) at P5.5. **D)** Fluorescent microscopy of the same brain as in panel C reveals robust but relatively localized (compare to Figure 1B) DsRed expressing cells. Note that injection site is visible (arrow). **E)** 200µm frontal section of zebra finch hatchling in panels A,B. The ventricle (V) is clearly visible (arrows). DsRed expression in the ventricular zone and in cells on the dorsal surface (DS) of the brain. **F)** DsRed expression in a 200µm frontal slice of the canary adult brain. Plasmids were targeted to the ventricle and show expression therein. Glial fibers are clearly visible (arrow). DS: Dorsal-most surface of the brain.



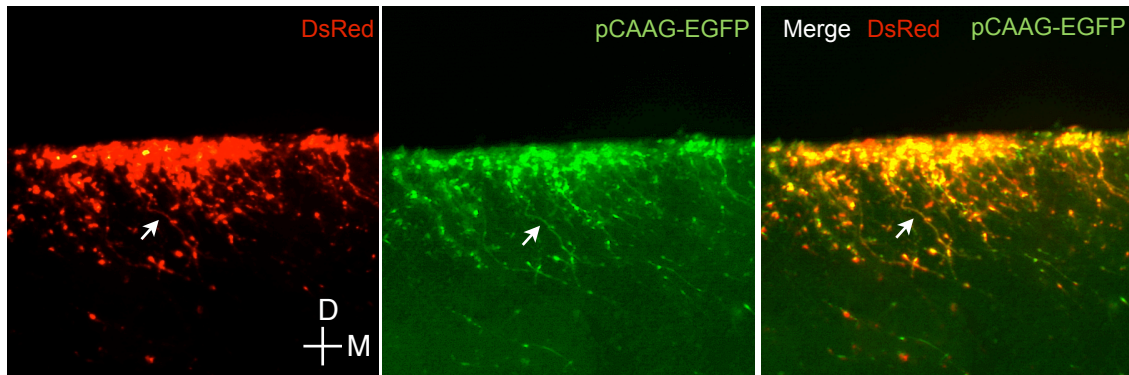


Figure 3: High co-expression of plasmids electroporated together.

Plasmids were mixed in a 1:1 dilution. **A)** pCAAG-DsRed expression in the adult canary ventricle. **B)** pCAAG-GFP expression in the same frontal section. **C)** High levels of co-expression across electroporated area. Note fine processes (arrow) to aid orientation.

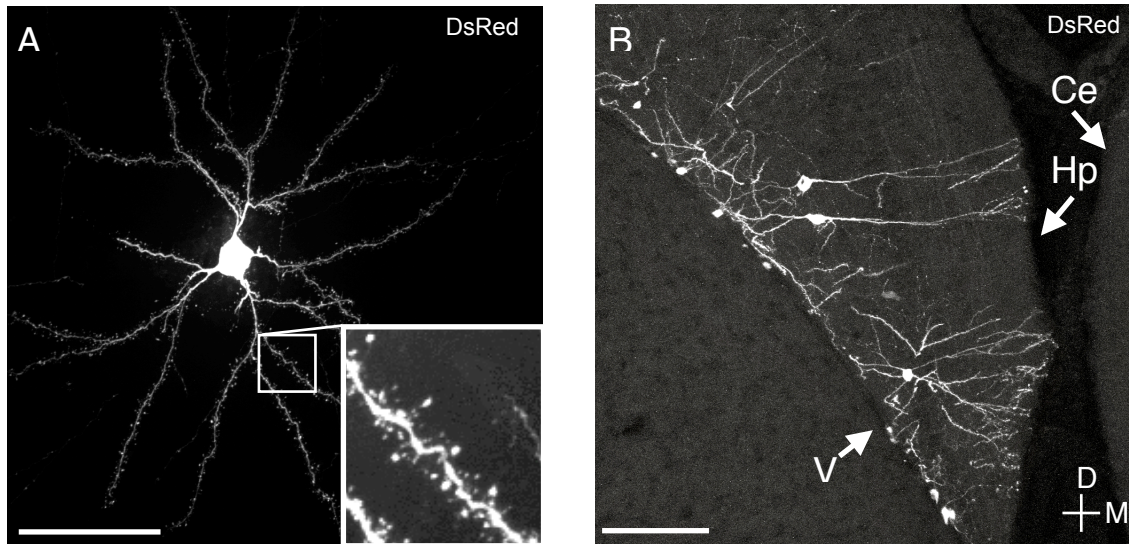


Figure 4: Gene expression is long lasting and can be directed. A) A large P111 day mature zebra finch neuron expressing DsRed that was introduced via electroporation at P3.5. Fine morphology is visible, including synaptic boutons (insert). **B)** Manipulation of electrode orientation successfully guides gene expression into the hippocampus. Frontal section with the dorsal ventricle (V) visible. Confocal (A,B) and light microscopy (C,D) images. Hp: Hippocampus, Ce: Cerebellum. Scale bar is equal to 50µm.

Successful expression of transgenes in a wild bird species.

Eastern Phoebe hatchlings (P3.5-5.5) were collected from the field, electroporated in the laboratory and hand-reared. Two phoebes were then anesthetized and perfused at ~P28.5 and found to contain robust areas of gene expression (Figure 4). Like electroporated hatchlings, swaths of cells throughout the ventricle were DsRed positive, with glial fibers visible (Figure 4B). Additionally, mature neurons were labeled (Figure 4A) throughout the nidopallium.

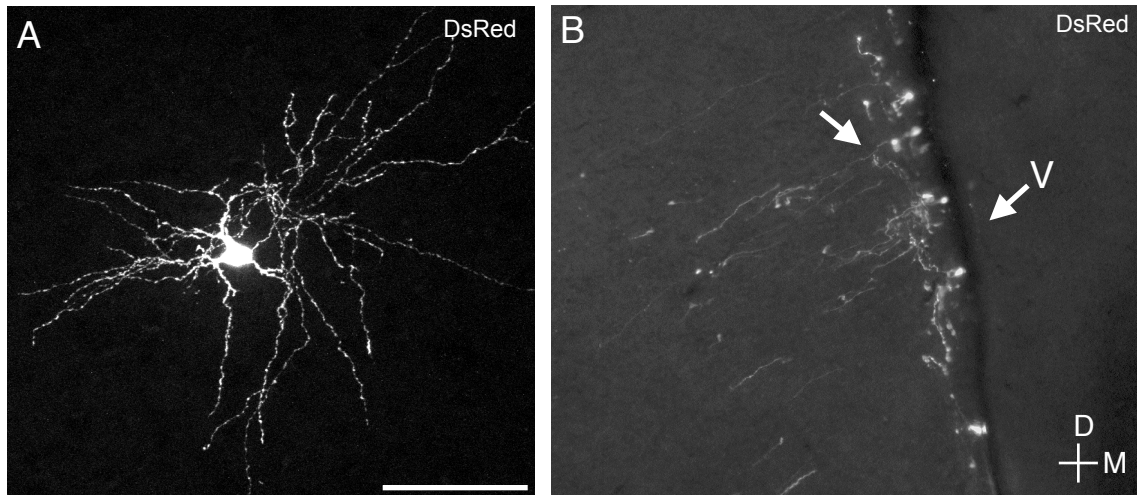


Figure 5: Electroporation from Eastern Phoebes. A) Mature DsRed positive neuron in the nidopallium. B) Strong DsRed labeling along the ventricular zone, with glial fibers visible (arrow). V = ventricle. Scale bar equal to 50μm.

Electroporation does not affect adult song or rearing behavior

To test whether electroporation had effects on adult song, we used SAP software to analyze similarity measurements for zebra finch songs produced before and after electroporation. Pre-electroporation (Pre) songs were compared to themselves (Pre-Pre) to serve as a control for baseline similarity measurements. Pre-electroporation songs were then compared to post-electroporation songs (Pre-Post) to measure changes in song due to electroporation. A one-way ANOVA revealed that there were no significant differences between Pre-Pre and Pre-Post songs in Percent Similarity, $F(1,6) = 0.157$, $p=0.70$, Mean Accuracy,

$F(1,6) = 0.35$, $p=0.86$, or Sequential Match, $F(1,6) = 1.223$, $p=0.28$, (Figure 6, Sequential Match not shown).

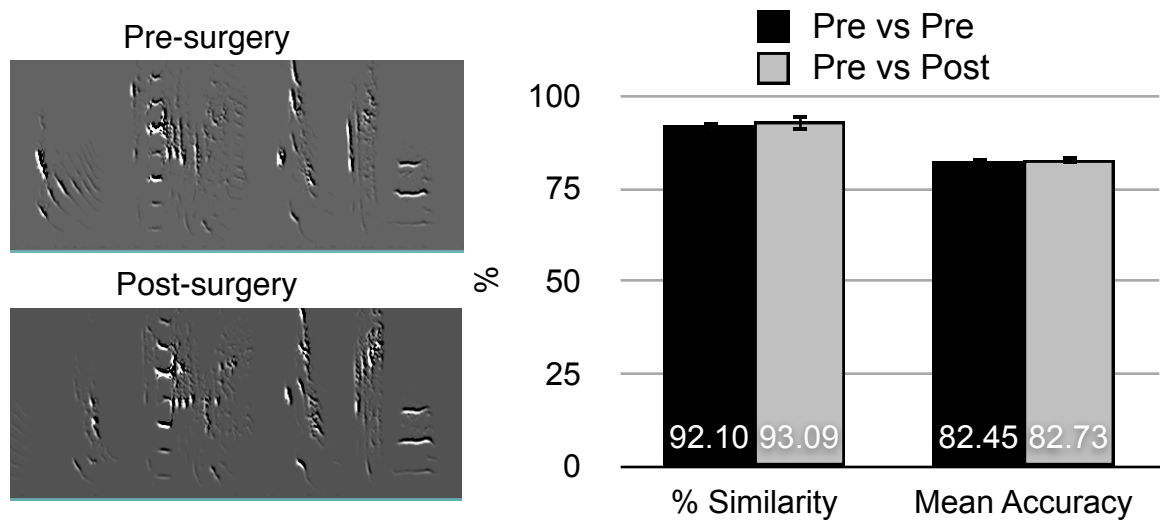


Figure 6: Electroporation does not effect song. Within individual zebra finches, songs before electroporation were compared to each other (Pre vs Pre) and then compared to songs following electroporation (Pre vs Post) using SAP. Presurgery songs compared to each other, and thus a reflection of intra-song variability, did not differ from comparisons with post-surgery song in either *% Similarity* nor *Mean Accuracy* scores.

To test whether electroporation had effects on more general behavior, we assessed breeding behavior in females. Seven adult female canaries were electroporated and seven control females were handled similarly but received no current. All females were immediately paired with adult males and allowed to breed. A one-way ANOVA revealed that there were no significant differences between control or electroporated females in the total number of eggs laid, $F(1,7) = 0.226$, $p=0.64$, the number of days taken to lay all eggs, $F(1,7) = 1.340$, $p=0.27$, the number of eggs hatched, $F(1,7) = 0.255$, $p=0.62$, the number of hatched offspring reared to P7, $F(1,7) = 0.094$, $p=0.77$, or the number of hatchlings having to be transferred due to poor health, $F(1,7) = 2.077$, $p=0.18$, (Figure 7). Additionally, all female birds, control or electroporated, constructed nests and no obvious differences in nest construction between groups was observable.

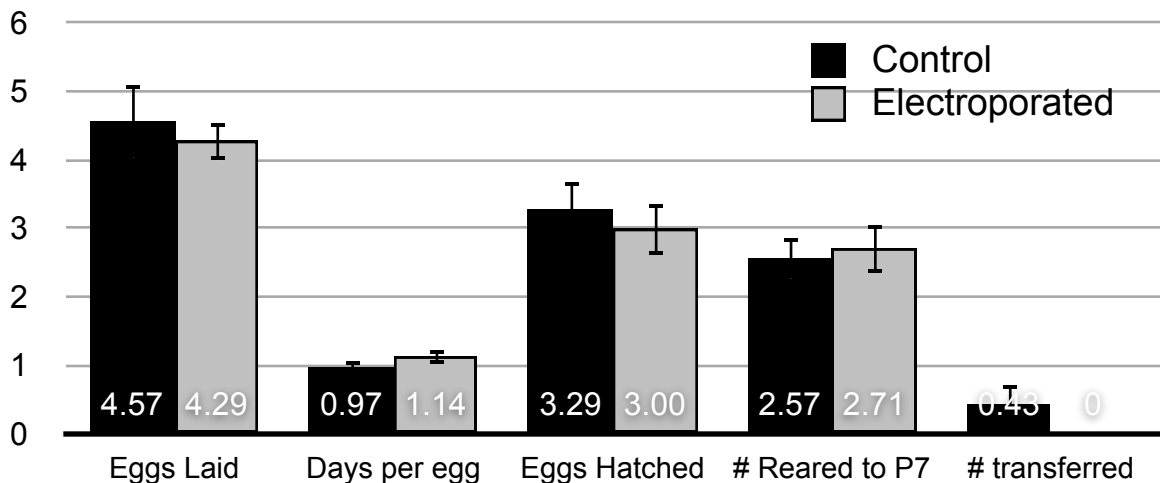


Figure 7: Electroporation does not effect rearing behavior in adult

female canaries. Electroporated female canaries (n = 7) do not significantly differ from control females (n = 7) in the amount of eggs laid, average amount of days needed to lay an egg, the number of successfully eggs hatched, the number of hatched chicks that are successfully reared to P7, or the number of hatchlings needing to be transferred to another nest due to ill health.

Conclusions:

The manipulation of gene expression in songbirds currently relies solely on the use of viruses (Agate et al., 2009; Haesler et al., 2007; Schulz et al., 2010; Scott & Lois, 2007). While viral-mediated gene transfer is an effective tool, it also requires special safety facilities and equipment, is costly, and has numerous technical limitations. Here we describe a rapid, highly efficient, cost effective, and potentially widely available method to drive gene expression in the brain of a variety of songbird species at various stages of development. Importantly, electroporation ameliorates some of the disadvantages of current viral-mediated gene transfer techniques in two regards, technical ease and genetics. Technically, electroporation is a simple and safe tool requiring relatively inexpensive tools. Perhaps more importantly, electroporation achieves robust expression across tissue and the plasmids used are simple to purify, can be very

large, and can be used in conjunction with other plasmids allowing for complex genetics without necessitating crossing multiple animals to achieve desired genetic combinations.

We describe a variety of electroporation methodologies that will allow researchers to alter gene expression to best suit experimental needs. Electroporation with the use of paddle-like electrodes resulted in robust, but preferential expression of genes in the ventricular zone (Figure 1E,F). In all animals electroporated with paddle-like electrodes, no transgene expression was ever observed in deep tissue. While we did not test why gene expression under this electroporation paradigm was preferentially targeted to the ventricular zone, we hypothesize that current passing through the brain preferentially moves across cerebral-spinal-fluid-filled ventricles due to lower electrical resistance. Nevertheless, this observation allows for great targeting accuracy of gene manipulation within the ventricular zone. Songbirds, which have served as powerful models for the study of neurogenesis (Alvarez-Buylla & Nottebohm, 1988; Alvarez-Buylla, Theelen, & Nottebohm, 1990; Barkan, Ayali, Nottebohm, & Barnea, 2007; Goldman & Nottebohm, 1983; Nottebohm & Liu, 2010a), may thus be more easily utilized for functional genetic studies. Additionally, the neurogenic lateral ventricle overlying HVC, a critical nuclei for song production, lies only 300-500µms below the dorsal surface of the brain and serves as a potentially powerful model for visualizing the birth and incorporation of new cells into functionally distinct circuits using deep-tissue microscopy (Harvey, Yasuda, Zhong,

& Svoboda, 2008; Holtmaat et al., 2009). We additionally observed that, under similar injection protocols, as birds aged the transgene-expressing area decreased (For example, compare Figure 2B to 2D). We here only used a 1.5 minute pause between plasmid injection and electroporation and it is possible that if given more time, plasmids could more widely diffuse in the ventricle. While the age-related result is possibly only due to the narrowing of the lateral ventricular zone as the brain develops, this observation can also be utilized by investigators to better aid in the design of experiments. For example, whole-hemispheric disruption studies could employ younger birds while more targeted ventricular fate-mapping studies might utilize older birds.

The orientation of electrodes used can also be altered to guide gene expression across a variety of brain areas. Manipulation of paddle electrode orientation allows for gene expression to be guided dorsally to the hippocampus for example (Figure 4B), but the same principle could be utilized for any particular tissue of interest. We did not experiment with deep tissue injections followed by needle electrode guided electroporation but this technique has recently been demonstrated in chickens (cite) and should be transferable.

Vocal learning, the ability to learn vocal signals using auditory feedback, is foundational for human language. While no non-human primates have yet been shown to learn their vocalizations, this ability has been described in cetaceans(Reiss, McCowan, & Marino, 1997), bats (Boughman, 1998),

hummingbirds (Baptista & Schuchmann, 1990), parrots (Pepperberg, Sandefer, & Noel, 2000), and, most thoroughly, in songbirds (Boughman, 1998; Nottebohm & Liu, 2010a; W. H. Thorpe, 1958; W.H. Thorpe, 1958). We sought to test whether electroporation-mediated gene transfer in hatchlings could remain throughout song development, thus allowing for gene manipulation during vocal learning. In zebra finches, song crystallizes when adults reach sexual maturity at ~90 days of age and thus zebra finch hatchlings electroporated at P3.5 were collected at P110-P111. All birds collected had robust gene expression 107+ days post electroporation (Figure 3A). As many different songbird species are used for the study of vocal learning (Liu & Nottebohm, 2007; Liu et al., 2009; Nottebohm, 1970; Podos, Nowicki, & Peters, 1999), any technique would have to be widely translatable. We here publish protocols for the manipulation of gene expression in two commonly used laboratory species as well as a wild-caught species. Canaries, zebra finches, and Eastern phoebes all successfully and robustly expressed transgenes following electroporation (Figures 2-5). Moreover, electroporation at specified voltages and ages (Table 1,2) does not appear to affect the health or behavior of songbirds significantly. Lastly, and critically, we demonstrate that electroporation does not alter song in adult males or cause disruptions in a variety of breeding related behaviors in adult females (Figures 6, 7). Thus, we present electroporation as a safe, widely adoptable and powerful tool for the manipulation of gene expression across songbird species, sex, age, and throughout various brain tissue types.

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