THE UNIVERSITY OF CHICAGO

SEX-SPECIFIC SYNAPTIC SPECIALIZATIONS SUPPORTING AND SUPPRESSING

SONG

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JOSEPH VINCENT GOGOLA

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This dissertation is dedicated to my grandmother, Ann, for all her unflappable support.

She unfortunately passed away in the year preceding its completion.

The pain slowly fades, but the love is forever.

The road to wisdom? –

Well, it's plain and simple to express:

Err

and err

and err again,

but less

and less

and less.

- Piet Hein

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An old Italian proverb, "*Alla fine del gioco, re e pedone finiscono nella stessa scatola*", roughly translates to, "Once the game is over, the King and the Pawn end in the same box." The work in this thesis, and my growth as a scientist and a human being, would not have been possible without the support of a great many people, all of whom have embodied this adage.

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Abstract

Male and female bodies are both sexually specialized, yet studies of sexual dimorphism predominantly focus on male specializations in ornamentations, brain circuits, and behaviors. The same is true of birdsong in the zebra finch (*Taeniopygia guttata*), where males learn to sing complex vocalizations, called songs, while females do not. Most work has focused on the specializations that males might have, and that females might lack, to promote singing.

Other recent work indicates that song is widespread and ancestral across songbirds and more recently lost in the female zebra finch, and partially regulated by sex hormones. These theories are supported in part by well-documented brain circuit differences between males and females. However, relatively less attention has been paid to the syrinx, the songbird vocal organ analogous to the human larynx, and the ultimate output of brain circuits controlling vocal behavior. In particular, the female syrinx has been vastly understudied, and this gap prevents understanding potential synaptic differences underlying the sexually dimorphic vocal behavior in this species.

In this thesis, I examined synapses and other neuromuscular features in the syrinx of males and females. First, I examined these features in adults, finding a surprising sex difference that is not male-biased, but instead female-biased. The adult female syrinx uniquely has a predominance of weaker *en grappe* synapses, which do not form a typical central motor endplate band in the muscle, and are biased towards smaller and weaker muscle fibers. Drawing on other well-studied models, these results suggest that female syrinx synaptic peculiarities might be an anatomical correlate of song suppression in this species.

Second, I asked how these adult sexual dimorphisms develop, and whether one sex is specialized at the start relative to the other, or if they both specialize with sex-specific

developmental programs. I find that both sexes have a common starting point of neuromuscular systems, and during developmental song learning, there is a divergence of these features. This implies two unique developmental programs leading to each adult phenotype, and further argues that both sexes are specialized at the level of syrinx synaptic morphology, likely in service of enhanced song production in males, and relative song suppression in females depending on species.

Third, I manipulated hormonal systems starting early in life to alter singing behavior, i.e., to suppress male song learning and production, and to spur females to learn and produce songs. This data is preliminary and descriptive in nature, but is intriguing for at least two reasons. First, it indicates the morphology and organization of vertebrate-typical *en plaque* synapses is tied in large part to song production. Second, it implies that song suppression is particularly tied to the presence of *en grappe* synapses outside the motor endplate band region of the vocal organ.

Altogether, these results broadly argue that the female synaptic specializations are the anatomical substrate of song suppression in zebra finches. Specifically, my data suggests that the location of *en grappe* synapses, either in endplate bands or out, is the strongest correlation to singing. They also suggest a mechanism by which an evolutionary loss of behavior may ultimately be realized at the level of synapses.

Chapter I: Introduction

Historical Perspective

"We should venture on the study of every kind of animal without distaste; for each and all will reveal to us something natural and something beautiful." In the 3rd century BCE, centuries before Darwin's theories, Aristotle described and classified the animals in his surrounding natural world. His extensive works, including History of Animals, Generation of Animals, Movement of Animals, and Parts of Animals (the latter of which includes the above quotation), were among the first to describe the anatomy and physiology of animals, the mechanisms of their movement, and the relationships between structure and function across species. He developed the theory of neuronal control of muscles which generate movements via contractions, and that different types of muscles have specific functions.

Theories on the relationship between muscles, their use, and behavioral output across species were further expanded by Sir Charles Darwin, particularly in the context of adaptive evolution. In his seminal work, *On the Origin of Species*, Darwin explored the connection between an organism's physical traits, including muscles, and their adaptive behaviors, observing that different species displayed specific behaviors well-suited to their environments. He studied the behavior of various animals, birds perhaps most famously, and noted how their specialized muscles and body structures enabled them to perform specific actions, such as flight. Darwin's work on sexual selection delved further into the link between muscles and behaviors, particularly in the context of courtship displays and mating rituals in animals. He suggested that certain behaviors, such as elaborate displays or contests between males, are for attracting mates or competing for reproductive success, and involve the coordinated use of specific muscle groups to perform

complex actions. By this time, science had come to accept that muscles were controlled by nerves sent from the brain, not Aristotle's cardiocentric idea of the heart as the main innervating organ. However, Darwin could only theorize about the ways and substrates by which the brain directed the muscles to produce behavioral output.

These ideas were quickly expanded upon by many contemporaries, which will be condensed for space. Emil du Bois-Reymond described electrical impulse properties of nerves and muscles (du Bois-Reymond 1843). Otto Loewi demonstrated this impulse transmission was chemical in nature (Loewi 1922). Sir Charles Sherrington recognized these systems involved connected neurons, and coined the connection point as a "synapse" (Sherrington 1906). Santiago Ramón y Cajal and his student Fernando Tello described the fine structure of this synapse, identifying the key components of this connection point, and deduced the direction of impulse transmission (Ramón y Cajal 1928). Sir Henry Dale introduced the term "neuromuscular junction" to describe this specialized synapse, identifying acetylcholine as the chemical responsible for mediating impulse action (Dale et al. 1936). Sir Bernard Katz showed that acetylcholine release was vesicular and quantal in nature, and described the localized density of postsynaptic receptors we today refer to as a "motor endplate", the site of impulse action (Del Castillo and Katz 1954). Sir Andrew Huxley deduced how these impulses led to muscular contractions (Huxley 1957). Viktor Hamburger described the development of neuromuscular systems during early embryonic life (Hamburger 1963). Rita Levi-Montalcini discovered nerve growth factor as a key molecular mediator of this development (Levi-Montalcini 1964).

Neuromuscular junctions are one of the longest studied and best understood vertebrate synapse. Much of what we know about the dynamics of neuronal connectivity, synapse formation,

and synaptic transmission in the brain was originally demonstrated at vertebrate neuromuscular junction, whose function is essential to the muscular contraction underlying all behaviors.

Current Perspective

All behaviors rely on muscles. Behaviors, as relatively simple as eye blinking or as relatively complex as singing operatic arias, universally require muscle activation to initiate, maintain, and cease those motor outputs underlying behavior. While behaviors may differ for many reasons, e.g., differing genetic makeup, developmental stage, or environmental experience and learning, most behaviors result from specific patterns of neuronal connections in brains ultimately instantiated at synapses between motor neurons and muscles. Thus, one strategy for understanding the neurobiological basis of different behaviors is to better understand differences in the neuronal circuits that produce those behaviors. One logical place to start is to look for differences at the specific connections between the brain and the muscles: the neuromuscular junction.

The Neuromuscular Junction

The neuromuscular junction (NMJ) serves as a crucial connection point between the nervous system and skeletal muscles, playing a fundamental role in motor control and coordinated movement (Sanes and Lichtman 1999). The NMJ represents the site of communication between motor neurons and muscle fibers, facilitating the transmission of signals that ultimately lead to muscle contraction.

The principal components of the vertebrate NMJ include the presynaptic terminal, the synaptic cleft, and the postsynaptic membrane of the muscle fiber. The presynaptic terminal is rich in mitochondria and specialized spheres called synaptic vesicles, which store and release the neurotransmitter acetylcholine (ACh) in vertebrates (Sudhof 1995). The synaptic cleft is a small

gap that separates the presynaptic terminal from the postsynaptic membrane, typically surrounded by a non-neuronal cell, a perisynaptic Schwann cell (Mirsky and Jessen 1996). The postsynaptic membrane of the muscle fiber contains clusters of acetylcholine receptors (AChRs), densely packed at specific regions known as the motor endplates (Sanes and Lichtman 2001).

At the NMJ, the presynaptic terminal of a motor neuron releases ACh into the synaptic cleft. ACh diffuses across the synapse and binds to AChRs located on the postsynaptic membrane of the muscle fiber. This binding initiates a cascade of events, triggering an influx of ions into the muscle fiber, leading to depolarization and the generation of an action potential. This action potential propagates along the muscle fiber, ultimately resulting in the contraction of the muscle.

Skeletal Muscles

Skeletal muscles comprise different fiber types that possess distinct contractile and metabolic properties, allowing for a wide range of motor functions and behaviors (Ogata 1988). The two primary types of muscle fibers are slow-twitch (Type I) and fast-twitch (Type II) fibers. Slow-twitch fibers are characterized by high oxidative capacity and resistance to fatigue. They contract more slowly but have greater endurance, making them well-suited for sustained, low-intensity activities such as maintaining posture (Luna et al. 2015). Fast-twitch fibers, on the other hand, contract rapidly but fatigue more quickly. These fibers are further classified into subtypes: Type IIa fibers possess a balance of oxidative and glycolytic capacities, while Type IIb or IIx fibers have higher glycolytic capacity and are well-suited for generating rapid and forceful contractions (MacIntosh et al. 2006).

The distribution of muscle fiber types within an individual's muscles can be influenced by genetic factors, training adaptations, and hormonal regulation (Reggiani and Schiaffino 2020). Sex

hormones, in particular, play a role in shaping the muscle fiber composition, contributing to sexual dimorphism in certain muscle-related behaviors. For example, higher levels of testosterone are associated with a greater size and proportion of fast-twitch fibers and an enhanced capacity for forceful muscular contractions and movements (Dent et al. 2012). Conversely, estrogens may contribute to a higher proportion of slow-twitch fibers, and has been shown to have a negative effect on muscle fiber size in some models, though not on muscle fiber type proportion (Haizlip et al. 2015). However, the roles of estrogens in muscle function are far less studied, particularly in experimental work, and thus their role in these systems are much less clear.

The functionality and efficiency of the NMJ and its muscle are crucial for motor control and coordinated movement, and is one of the best examples of an "all or nothing" synapse - every electrical impulse from the presynaptic motor axon, in adults, leads to a postsynaptic action potential (Lucas 1905). Disruptions or abnormalities in the NMJ can lead to neuromuscular disorders and impairments in muscle function. Dysfunction in the release of acetylcholine or alterations in the density or function of acetylcholine receptors can result in muscle weakness, fatigue, and motor coordination deficits (Li et al. 2018). In adulthood, these innervations are one-to-one, meaning that each postsynaptic muscle fiber is contacted by one presynaptic motoneuron axon.

NMJs in Development

During embryonic development, the formation of NMJs is a complex process involving precise molecular and cellular events, and proceeding in tune with muscle development. Early in neonatal life, motor neurons extend axons toward their target muscles, guided by specific guidance cues (Lance-Jones 1988). As motor neurons approach their muscle targets, they interact with developing muscle fibers through a series of attractive and repulsive signals (Bonanomi and Pfaff

2010). These interactions trigger the formation of nerve terminals and postsynaptic clusters, marking the initial establishment of the NMJ. Following the initial formation of NMJs, synaptic maturation processes occur, allowing the NMJ to acquire its functional properties (Donoghue and Sanes 1994). The establishment of neurotransmitter release machinery, including the docking and fusion of synaptic vesicles, plays a crucial role in ensuring efficient synaptic transmission. Various signaling molecules, such as neuregulin and agrin, contribute to the maturation of both pre- and postsynaptic components, ensuring precise and coordinated synaptic communication (Sanes and Lichtman 2001).

During the early postnatal period, the neuromuscular junction undergoes activity-dependent refinement - baby muscle fibers receive inputs from multiple motor neurons, and adults often refine to a single input (Meirovitch et al. 2021). Neural activity, particularly spontaneous and evoked muscle contractions, plays a critical role in shaping NMJ structure and function. Hebbian-like plasticity mechanisms, such as long-term potentiation and depression, influence synaptic strength and stability, contributing to the establishment of functional motor circuits (Hogan et al. 2020). During this time, the proportion of twitch muscle fibers also increases, while the proportion of tonic fibers decreases, though this is muscle-dependent (Schiaffino and Reggiani 2011).

NMJs can occur along the length of muscle fibers during formation but maturation leads to, among other things, their refinement into a centralized motor endplate band ("MEB"; Purves and Lichtman 1985). The MEB is a region of the muscle where innervation is centralized, with NMJs very close to each other in proximity and forming a visual "band" of innervation across the muscle fibers, allowing for the greatest force and control of muscle contractions - and is a classic feature of fully formed adult innervation and normal muscle function. There is also local competition for NMJ space by motor axons, and these activity-dependent processes gradually shift

NMJs from being innervated by many axons, to being innervated by a single motor axon (Meirovitch et al. 2021). This process is essential to efficacious neurotransmission, muscle contraction, and, ultimately, meaningful behavioral output (Nguyen et al. 1998).

NMJs are typically considered static structures in adulthood, highly stable throughout the lifespan (Lichtman and Wilkinson 1987). The same is broadly true of muscle fiber types, which by adulthood are predominantly twitch fibers (Tieland et al. 2018). Indeed, NMJ components have been well studied in their relationship to muscle use, and there is a tight correlation across many models in the size of an NMJ, its muscle fiber, and the force production of that muscle (Balice-Gordon and Lichtman 1990; Boehm 2020). While there are many species differences in actual morphology of NMJs, as well as within-species differences across different muscles, NMJ sizes are typically tightly correlated to the size and type of the muscle fiber in vertebrates (Boehm 2020). This is due in large part to the NMJ being the main actuator of muscle function. Larger NMJs are positively correlated to larger and stronger muscle fibers, greater synaptic transmission, stronger synaptic connections, and greater muscle-specific force generation (Bass and Marchaterre 1989; Jones 2016; Jones 2017; Kasthuri and Lichtman 2003; Lichtman 1985; O'Bryant and Wade 2002). Thus, NMJs are poised to be an informative piece of anatomy with which to view differences in behavior within a species.

NMJ Classes in Vertebrates

Two primary classes of NMJ synapses are recognized: the 'classic' vertebrate *en plaque*, and the less common, *en grappe* synapses. These classes differ in size, morphology, prevalence, and functional properties of their postsynaptic targets (Kasthuri and Lichtman 2003; Wilkinson and Lichtman 1985). They have been extensively studied in various vertebrate models, though the

en plaque NMJs are the most well studied class, and from where the majority of our NMJ knowledge arises.

With few exceptions, the vast majority of vertebrate muscles studied typically have only one NMJ class within a muscle, usually *en plaque*, though differences in class or morphology across muscles are also possible (Fox 2011; Lichtman 1985, Mech et al. 2020). This is thought to be influenced, in part, by the activity of motoneurons from which they receive their innervation (Kasthuri and Lichtman 2003; Jones 2016; Jones 2017). *En plaque* NMJs are singly-innervated, while *en grappe* NMJs are typically multiply innervated. There is some evidence to suggest that *en grappe* NMJs do not undergo synaptic pruning, perhaps due to postsynaptic muscle fibers firing graded versus regenerative action potentials (Lichtman et al. 1985, Lichtman and Wilkinson 1985). Moreover, there is species-specificity in NMJ class within the same muscles, with individual behavioral differences more closely tied to differences in NMJ synaptic strength or transmission than variation in NMJ class (Mech et al. 2020; Boehm et al. 2020). Differences in these morphological features of NMJs have particular consequences for muscle function; for instance, an NMJ with a larger surface area enhances synaptic efficacy by allowing more synaptic vesicle docking and release at a single moment, leading to a stronger and more reliable muscle contraction (Sanes and Lichtman 1999). Thus, NMJs are a useful substrate with which to assess variability in behavioral output.

Mammalian en plaque and en grappe Synapses

In mammals, including humans, the NMJ synapses primarily fall into the *en plaque* category. *En plaque* synapses are characterized by their large, complex, and flattened morphology, taking their name from their plaque-like appearance. Presynaptic nerve terminals are directly opposed to postsynaptic AChRs at all locations, called the motor endplate, which extensively

contacts the postsynaptic muscle fiber membrane (Sanes and Lichtman 1999). *En plaque* synapses are found on twitch muscles, or the Type II fast and superfast muscles classes. Synaptic transmission at *en plaque* NMJs is incredibly reliable, as single presynaptic action potentials 100% of the time lead to postsynaptic spikes and muscle fiber contraction. In contrast, *en grappe* synapses are less prevalent in mammalian NMJs but have been observed in certain specialized muscles, such as the extraocular muscles of rodents, cats, et al. (Fox et al. 2011; Khanna et al. 2003; Porter et al. 2002) *En grappe* synapses consist of multiple smaller nerve terminals that cluster around the muscle fiber, resembling a bunch of grapes or beads on a string. They tend to be found on tonic or non-twitch muscles, the slow muscle fibers (Luna et al. 2015). These muscles tend to have lower muscle-specific force generation, slower contraction speeds, and no bursting activity (Lichtman et al. 1985; Lichtman and Wilkinson 1985). These synapses putatively provide a broader innervation pattern and are thought to be involved in potentially controlling broader muscle groups or exerting more generalized motor control, though due in part to their infrequency, there is a paucity of evidence to make a strong claim therein.

En grappe Synapses in Non-Mammalian Vertebrate Models

Beyond mammals, *en grappe* synapses are more commonly observed in non-mammalian vertebrates. Snakes, such as the garter snake (Thamnophis sirtalis), have been well-studied for their occurrence of *en grappe* NMJs in their transverse abdominal muscles (Lichtman et al.1985; Lichtman and Wilkinson 1985). Here, *en grappe* synapses are multiply innervated and solely found on tonic fibers, in the same endplate band region as the twitch-specific *en plaque* synapses. Similarly, reptilian and amphibian species also exhibit *en grappe* synapses with similar organizational properties, i.e., found on tonic fibers (Mackay et al. 1960; Lichtman 1985; Ridge 1971).

In contrast, fishes, such as the Sea Horse (*Hippocampus erectus*; Bergmann 1967), Northern Snakehead Fish (*Channa argus*; Nakajima 1962), and Common Carp (*Cyprinus carpio*; Nishihara 1966), exhibit *en grappe* synapses as the predominant NMJ class on all muscle fiber types, even on twitch fibers. Here, multiple nerve terminals converge onto a single muscle fiber, forming distinctive *en grappe* structures strung out along a large length of the muscle fiber. Similarly, in birds, the chicken (*Gallus gallus*) iris has been reported to follow a similar organization, where the muscle fibers are predominantly twitch fibers, but the synapses are of the *en grappe* type (Hess 1961). In all these cases, the occurrence of *en grappe* synapses on twitch, not tonic, muscle fibers brings a point of contention as to whether functional properties of NMJs can be denoted simply by their morphology. *En grappe* synapses generally have a smaller surface area than *en plaque* synapses, a lower packing density of AchRs, and are often located on smaller muscle fibers than *en plaque*, indicating a putatively weaker synapse. Further functional studies would be required to make stronger claims, but I argue there is good standing to assert that an *en grappe* synapse on a twitch muscle is a weaker synapse than an *en plaque* synapse on a similar muscle.

The Zebra Finch (*Taeniopygia guttata*) Songbird as a Model

Biological variation in how motor neurons connect with muscles is tied to differences in muscle function and subsequent behavior across a wide range of taxa. However, most behaviors studied at NMJs are not about variations amongst individuals, particularly not in individuals of the same species whose behaviors differ dramatically. Such data would elucidate how foundational properties of NMJ physiology can either facilitate or be a consequence of stark differences in fundamental behavioral output.

The zebra finch songbird model is a useful lens to investigate the relationship between neuromuscular connectivity and behavioral variation within a species. Males sing complex songs, highly stereotyped and actively maintained in adulthood; females do not produce learned song vocalizations, but primarily unlearned call behavior, much shorter in length and spectrally much less complex than song (Roper and Zann 2006). Both sexes vocalize using a set of muscles called the syrinx, analogous to the human larynx (Kingsley et al. 2018). Much is known in this species about the gross muscular morphology of the vocal organ and the dynamics underlying song production (Adam 2021; Bush 2018; Mindlin and Laje 2005; Riede and Goller 2010; Srivastava et al. 2015). Though both sexes have the same set of syringeal muscles making up the vocal organ, there exist numerous sex differences in their component parts. Adult males have larger overall syrinx weight and muscle fiber size than females, a greater number of syringeal muscle fibers, and a different complement of muscle fiber types (Wade and Buhlman 2000; Wade et al. 2002; Christensen et al. 2017). In addition, recent work has shown the adult male syrinx has all the molecular machinery necessary to synthesize its own androgens locally, instead of relying solely on gonadal hormones, indicating there might be local fine-tuning of hormonally mediated structure and function (Schuppe et al. 2022). Finally, despite our understanding of differences at the level of muscles and behavior, the relatively small number of studies describing NMJs are either more focused on molecular markers, or on males alone (Bleisch et al. 1989; Faunes et al. 2017).

Much is unknown about potential differences in NMJs in male and female syringes, and how these differences might support sex-specific behavioral outputs. The study of NMJ structure in zebra finches allows us to take advantage of naturally occurring, sexually dimorphic, and ethologically relevant learned behavior, giving a unique opportunity to examine how peripheral connectivity is particularly tuned in service of specific complements of behavioral output.

Sexually Dimorphic Adult Vocal Behavior

Adult zebra finches exhibit one of the most extreme sexually dimorphic vocal behaviors in the animal kingdom. Males make complex learned vocalizations called "songs", spectrally rich and containing different subunits (syllables) often of distinct categories (e.g., sweeps, high notes, or stacks). As adults, these songs are incredibly stereotyped, with each male singing the same sequence of the same syllables across hundreds to thousands of renditions each day and hundreds of thousands of renditions across adult life, unchanging without experimental perturbation (Zann 1996). Thus, the brain and body circuitry underlying these song behaviors are broadly unchanging across adulthood, which has been shown at varying levels of the biology (James and Sakata 2019; Pytte 2007; McDonald and Kirn 2012). Moreover, every adult male has an individual song, and typically no two songs are exactly alike, allowing (in part) its use as a vocal signal for individual recognition.

In contrast, females do not produce these complex learned songs, but primarily unlearned single-note vocalizations termed "calls", shorter in length and spectrally much less complex than male learned songs. While there is emerging evidence that females may learn to produce calls in differing contexts and based on the vocalizations of a conspecific, termed predictive call timing, the exact spectral content of those calls are considered unlearned (Benichov et al. 2016). There are at least 11 types of calls that both males and females produce in differing contexts (Elie and Theunissen 2016, 2018; Zann 1996). However, the female vocal behavioral repertoire is, in some measure, more complex than males, whose brains and bodies are primarily tuned for singing behavior (Carouso-Peck and Goldstein 2019; Hauber et al. 2021; Mainwaring et al. 2011; Prior et al. 2019; Nottebohm and Arnold 1976). While both males and females are able to produce a similar variety of vocalization types, females utilize their full vocal range more often than males, whose

primary vocalization is song (Elie and Theunissen 2016; Elie 2011). Females tend to produce louder and longer duration distance calls, and longer duration and higher frequency *tet* calls, than their male counterparts (Loning et al. 2022; Zann 1984; Elie et al. 2016). Similar to male songs, female calls can also be used as a vocal signal for individual recognition across both sexes. Identity coding has been shown for at least 4 call types independently, and for the sum of the vocal repertoire, due in part to the high individual stereotypy of these non-song vocalizations (Elie and Theunissen 2018).

The benefits of these behaviors are therefore at least twofold. First, an unchanging behavior means that any bird at any adulthood age is comparable to any other - a bird who has just reached adulthood is putatively highly similar in terms of vocal behavior and its underlying biology as that same bird nearing senescence, despite accumulated adulthood experience. Second, due to the highly individual learned song, comparing across adult males is a useful method to uncover potential biological variability that contributes to those individual differences, which has already been shown at some levels of the biology (Daou and Margoliash 2020, Daou and Margoliash 2021). Thus, studying zebra finches in the context of vocal behavior is a fruitful area of study to map individual variation in cells, circuits, and anatomy to both aspects of vocal learning and eventual variation in vocal behavior, both at the individual level within sex, and between sexes.

Sexually Dimorphic Development

Adult male and female bodies are both specialized, yet early in development, these specializations have often not yet emerged. Adult specializations typically arise over time from multiple complex processes - genetic encoding, developmental programs, experience - and with varying degrees of overlap therein. Thus, there are multiple potential levels of regulation that may lead to adult sexual dimorphisms. One possibility is that, early in development, both sexes are

highly similar or sex-neutral, after which one sex develops a specialization, and the other sex fails to do so. This would imply that the "non-specialized" sex in adulthood would be indistinguishable from either sex early in development - or, in other words, an undeveloped or underdeveloped adult. Competing theories argue that, early in development, either sex has the potential to develop a behavioral specialization, and their adult phenotypic differences represent the outcome of two active developmental processes, in one sex promoting behavioral development, and in the other suppressing that development.

The zebra finch model provides an excellent opportunity to address these theories in the context of vocal behavior. Zebra finch vocal behavior is sexually dimorphic in adulthood - males sing complex learned songs, while females do not (Roper and Zann 2006). As with many adult behaviors, male song is primarily learned during early post-hatch development, through a confluence of multiple overlapping learning programs. Juveniles must first hear and memorize the song of an adult male during a sensory learning phase (Immelmann 1969). Soon after, juvenile males begin to make song-like vocalizations, often called "plastic song" or "vocal babbling" due both to its similarities to early human vocalizations and dissimilarities from adult stereotyped vocal behavior (Goldberg and Fee 2011). At this time, juvenile males explore song space through a process of trial-and-error as they learn to turn their vocal intent into motor action (Olveczky et al. 2005; Ravbar et al. 2012). This phase is called sensorimotor learning because juvenile males are putatively comparing their motor output to the sensory memory trace of the song model, though the exact specificity and location of this song model in the brain has not yet been elucidated. By the end of this learning phase, coinciding with reproductive maturity, the song is fully crystallized and remains unchanged in adulthood (Mooney 2009). In this way, the zebra finch is an "age-limited" learner, as despite constant exposure to numerous different songs throughout life, these

experiences do not continue to influence their own vocal production after the developmental song learning period has ended (Eales 1985). Thus, synaptic differences that may arise during development should be informative to understanding the eventual stable adulthood vocal behavioral and synaptic sexual dimorphisms.

Development of zebra finch song circuitry between males and females has been extensively studied. Females possess the similar song circuit nuclei as males, but they are generally smaller, with fewer cells, more simplified neuronal processes, and diminished or absent connectivity between nuclei (Arnold and Nottebohm 1976; Shaughnessy et al. 2019). How these sex differences emerge is also developmentally regulated. In the songbird premotor cortex analogue, HVC (used as a proper name), the volume increases in both sexes prior to the onset of developmental song learning (P25), though this relative increase is smaller in females relative to males (Bottjer et al. 1985). By the middle of the sensorimotor learning phase (P53), the male HVC has further increased in volume, while it has moderately decreased in females. This is the same general timeline of development in the motor cortex analogue RA (robust nucleus of the arcopallium) for both males and females, though here the increase in male size is more robust between P25 and P53. This is thought to be due, in part, to the fact that HVC and RA do not become synaptically coupled until between P30-P40 (Konishi and Akutagawa 1985). Interestingly, in nXIIts (the twelfth cranial nerve), one of the main outputs of RA and the motoneuron pool innervating the syrinx, the developmental sex differences are somewhat more moderate. While the volume of the nucleus grows moderately larger in males than in females around P20-30, the neuronal density and total number of neurons in this nucleus is not sexually dimorphic, even into adulthood (Godsave et al. 2002). In addition, adult males and females have similar intrinsic properties in these motoneurons, implying that sex differences in the syrinx may not be wholly driven by physiological properties

of their innervating motoneurons (Roberts et al. 2007). But overall, the consensus is that, in the male brain, song system circuitry grows larger and more complex during development and in support of vocal learning, while in the females, they become anatomically atrophied. Whether the same ontogeny extends to the syrinx is unknown.

Hormonal Sensitivity in the Zebra Finch

Hormonal regulation of the brain, body, and behavior is complex in songbirds. In the periphery, males have testes that produce high levels of androgens to circulate through the rest of the body, while females have ovaries that produce high levels of estrogens, similar to mammals. However, in the brain, estrogens are synthesized locally and are thought to have an organizational effect in males, being necessary for song system development and vocal behavior. In in vitro slice culture preparations, the development of the male-typical robust HVC-RA connection can be spurred in females by estrogen exposure, and prevented in males by estrogen antagonism (Holloway and Clayton 2001). The same is true *in vivo*, as females treated with estrogens during early development grow to be adults with masculinized plumage, brains, and singing behavior (Simpson and Vicario 1991a; Simpson and Vicario 1991b). Attempts to demasculinize the male song system by hormonal treatment have been more mixed. Reduction of estrogen signaling via either inhibition of aromatase, the enzyme that synthesizes estrogen from androgens (Merten and Stocker-Buschina 1995), or antagonism of G-protein coupled estrogen receptors (Tehrani and Veney 2018) show moderate reductions in cell soma size and circuit nuclei volumes, but seem to have no effects on song behavior. More recent reports (Choe et al. 2021) have shown that more efficacious aromatase inhibitors impair song learning in males, but have little to no effect on anatomical features of the song circuit or the specialized transcriptomes of individual nodes of this circuit. While it is far from clear what specific organizational roles hormones may have in driving

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sex-dependent brains and behavioral dimorphisms, it is clear that brains and behaviors of both sexes are broadly hormonally sensitive in a sex-dependent manner.

There is also evidence for hormonal sensitivity of the syrinx in the zebra finch. As adults, males have a syrinx that is around 1-2x the size of the female in terms of gross weight and muscle fiber size (Düring 2013). When adult males are either supplemented with estrogens, or have endogenous androgens reduced via castration, their syrinx weight is reduced; while androgen treatments do not increase the weight of the syrinx in normal adult males, they do return the syrinx to a normal male weight when castration-induced reduction on endogenous androgen levels is recovered by exogenous androgen treatment (Harding et al. 1983). Moreover, the adult male syrinx has been shown to have the molecular machinery necessary to synthesize its own androgens locally, instead of fully relying on hormones produced by the gonads (Schuppe 2022). This indicates the syrinx is not only sensitive to hormonal milieu, but that hormones play an integral role in maintenance and function of the syrinx across adulthood.

The adult female syrinx is similarly sensitive to hormonal milieu, though has been less studied than in males. Emerging evidence indicates the female syrinx is actively maintained via estrogens, and not that its features are simply not masculinized due to lower levels of circulating androgens. The female syrinx can be masculinized in both weight and amount of NMJ synaptic components by ovariectomy, i.e., reducing endogenous estrogens, and supplementing with exogenous androgens (Bleisch et al. 1984). Treatments of females with aromatase inhibitors (the enzyme that converts androgens into estrogens) has been shown to lead to an intermediate increase in syrinx size, i.e., a female syrinx that is larger than a normal female. In addition, androgen supplementation of adult females increases both syrinx size and superfast muscle fiber percentage (Allred et al. 2011), though that treatment alone is not enough to induce song-like vocalizations.

Thus, there is a complex interplay between hormonal milieu, muscle use, and behavior that is unlikely to be fully uncovered by only studying adults.

The developmental hormonal sensitivity of the zebra finch syrinx is a particularly useful framework to answer these questions. In this species, hormonal treatment early in development has been shown to induce sex-reversal at some levels of the biology and behavior, most strikingly in singing behavior. Juvenile females supplemented with estradiol (the main bioactive estrogen; "E2") throughout development are capable of developmental song learning, producing male-like learned songs as adults. They also develop male-like plumage and actively court adult females, despite their lack of functional testicular tissue. Interestingly, early E2 treatment does not seem to make the female syrinx more male-like in terms of weight or muscle fiber size at P60 (Wade et al. 2002), and there is anecdotal evidence of this lack of syringeal masculinization being maintained into adulthood, though data has not been reported (Simpson Vicario 1991a; Gurney et al. 1982). How synaptic components of the syringes of these masculinized females might be hormonally sensitive, and how they might change to support song production, is wholly unknown.

Similarly, juvenile males may be induced to become more female-like by developmental hormonal manipulations. Developmental treatment with an aromatase inhibitor has been shown to depress male song-learning ability and reduce male-like plumage, despite little to no effects on brain anatomy or gene expression (Choe et al. 2021). Similar work has also been shown to make male syringes more female-like, both in terms of syrinx weight and muscle fiber size, though at an intermediate level (Wade et al. 2002). Interestingly, similar treatments in females lead to a syrinx that is also an intermediate between normal males and females, i.e., a female syrinx that is larger than a normal female (Wade et al. 2002; Gong et al. 1999). However, similar to the female work

in the prior paragraph, hormonal sensitivity of synaptic components in feminized males, and how they might change to support repressed song learning and production, is also unknown.

Scope of this Dissertation

The basic synaptic features of the zebra finch vocal organ which converts motor signals into muscle movement and vocal behavior are poorly understood, and are clearly critically important for vocalizations broadly and song specifically. This is surprising, considering the extreme sexual dimorphism in vocal behavior this species exhibits. Two broad questions emerged here. First, is the sexual dimorphism in behavior, which is supported by neural circuitry differences in brains, ultimately instantiated at the synapses connecting those circuits to muscles underlying vocal behavior? Second, if such differences exist, do they emerge across development, perhaps locked to the emergence of, and/or driven by, sexually dimorphic vocal behavior? Such a conjunction between behavioral differences and synaptic morphology, if changing over development, would provide some clarity into the functional role of the vocal musculature in either promoting or suppressing vocal abilities.

I explored the first question using established techniques for visualizing pre- and postsynaptic components of NMJs and muscle fiber types in adult males and females. I sought evidence for synaptic specialization by viewing both the syrinx and non-vocal muscles. My results demonstrate that there is a sex difference in syringeal synaptic features, not necessarily in support of male song, but instead correlated with the lack of song in females - that is, a potential anatomical substrate for song suppression.

I explored the second question by quantifying NMJ type and distributions in males and female muscles at different ages of early life, anchored to important vocal developmental

milestones. I found clear evidence for a common starting point, and diverging developmental programs in both sexes, i.e., both sexes have their own synaptic specializations in the syrinx. This indicates that the adult sexual dimorphism is not a case of failure to develop on the part of the female, but an active development towards the adult stereotyped pattern, which might also support song suppression.

Finally, I attempted to address these questions by manipulating hormonal systems during development, to spur non-singing females to learn and produce songs, and to repress male singing ability. I observed striking changes to the basic synaptic morphology and organization of the syrinx and song in both cases, and though it is only preliminary data, indicates that *en grappe* NMJs specifically outside the canonical MEB region are positively correlated to song suppression. Taken together, this provides the groundwork for understanding the mechanisms and anatomical substrates by which an evolutionary loss of behavior might ultimately be realized at the level of synapses.

Chapter II: A Synaptic Correlate of Sexually Dimorphic Singing at Vocal Organs of the Zebra Finch Songbird (*Taeniopygia guttata*)

Abstract

Zebra finch singing is sexually dimorphic - adult males vocalize intricate songs while females do not. Such dramatic behavioral differences likely have correlates in neuronal wiring. Here, we report stark sex differences in motor neuron innervation of the muscles of song production, the syrinx. Male and female syrinx neuromuscular junctions (NMJs) differed both in degree - male NMJs were larger than females - and in kind, most male NMJs were classical *en plaque* synapses, while female NMJs had near-equal distributions of two NMJ types, *en plaque* and *en grappe*. We saw little sex differences in the innervation pattern of muscles unrelated to vocal song production, e.g., *latissimus dorsi*, even in muscles innervated by the same cranial nerve as the syrinx, e.g., the tongue. *En grappe* NMJs consistently localized to the smallest muscle fibers - predominantly weaker, oxidative, Type IIa, 'fast', muscle fibers rather than stronger, glycolytic, Type IIb, 'superfast' muscle fibers. *En grappe* NMJs did not form a canonical motor endplate band and we frequently saw branches of the same motor axon innervate both types of NMJs, indicating the female specialization in NMJ class does not correspond to specialization in motor neurons. Thus, we show clear sexual dimorphism in synapses specifically and directly related to song production in zebra finches. Recent models suggest song production is actively suppressed in zebra finch females, and the qualitative and quantitative sex differences at syrinx NMJs might be a synaptic correlate of such suppression.

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Introduction

Understanding how patterns of neuronal connections generate behavior remains a significant central challenge in neurobiology. One strategy has been to identifies behaviors that differ among species or individual animals to guide the search for corresponding variations in neural circuits. However, for many behaviors, the specific neurons and connections that directly mediate said behavior remain unknown and variability in individual behaviors compounded with variability in circuits makes such comparisons challenging (Asahina et al. 2022, Ruff et al. 2013, Neinborg et al. 2012). Additionally, until recently, large-scale analyses of neuronal circuit connections have been intractable (Kasthuri et al. 2015, Shapson-Coe et al. 2021, Foxley et al. 2021).

Analyzing neuronal circuits for sexually dimorphic behaviors can circumvent some of these confounds. Sexually dimorphic behaviors are archetypically distinct across sexes, even with variability in individuals of the same sex (Darwin 1871). They play central roles in reproductive success, e.g., mate selection, suggesting similar robustness in neural circuitry (Darwin 1871, Konishi 1985, Kelley 1988, Breedlove 1992). There are numerous experimental manipulations (including developmental (Simpson and Vicario 1991a, Choe et al. 2021), adulthood (Madison et al. 2015, DeAngelis et al. 2017, Gonzalez et al. 2021), hormonal manipulations and social context (Phillips et al. 2020, Woolley and Doupe 2008, Chen et al. 2016), and others) that can alter sexual dimorphic behaviors, allowing for investigations of causal mechanisms for differences seen in the underlying neural circuits.

Bird song production is an extensively studied example of sexually dimorphic behavior, although the neural mechanisms of male song are far more studied than are those of females (Nottebohm and Arnold 1976, Ball and Balthezart 2020, Brenowitz and Remage-Healey 2016). For example, male zebra finches (Taeniopygia guttata) learn and sing highly stereotyped,

complex songs which are actively maintained in adulthood (Immelman 1969, Zann 1996, Nordeen and Nordeen 1992). However, females produce primarily unlearned call behavior, shorter in length and less complex than male songs. Females participate in complex social interactions with males and other females where the timing of calls conveys information, and is likely to be learned (Benichov et al. 2016, Elie et al. 2010). This sexually dimorphic vocal behavior is extreme relative to other species of songbirds (e.g., doves (Ballintijn 1997), canaries (Nottebohm 1980), starlings (Pavloba et al. 2005), blackbirds (Whittingham et al. 1992), and many others (Odom et al. 2014)) or other non-avian vocalizing species (e.g. lizards (Chen et al. 2020, Jorgewich-Cohen et al. 2022, Labra et al. 2013), frogs (Leininger and Kelley 2015), singing mice (Campbell et al. 2014), bats (Suga et al. 1987), humans (Lieberman 1986)), which should facilitate its analysis.

Whereas brain pathways for learning and unlearned vocalizations have been described in the zebra finch, the description of the sexually dimorphic peripheral specializations are not as well explored. While male and female syringes (songbird voicebox) are composed of the same set of muscles, male syringes are heavier, have more and larger muscle fibers, and a different complement of muscle fiber types (Düring et al. 2013, Wade and Buhlman 2000, Wade et al. 2022, Christensen et al. 2017, Adam and Elemans 2020). However, less is known about sex differences at synapses between motor neurons and individual muscle fibers, neuromuscular junctions (NMJs). NMJs are profoundly impacted by differential muscle activation, behavioral complexity, and hormonal milieu (Smith and Rosenheimer 1982, Balice-Gordon and Lichtman 1990, Balice-Gordon et al. 1990, Valdez et al. 2010, Jordan et al. 1989a, Jordan et al. 1989b, Mech et al. 2020). Thus, I hypothesized that NMJs of male and female zebra finch syringes would also show sexual dimorphisms.

I characterized NMJ morphology, spatial distributions, and type of muscle fiber they innervate in the syrinx. I compared these features to muscles unrelated to vocalization (*latissimus dorsi)*, even when innervated by the same brainstem nuclei as the syrinx (the tongue). I show qualitative and quantitative sex differences in the syringeal muscles of the female, which have different classes of NMJ (*en grappe* NMJs), different spatial distributions of these synapses and fiber types absent in all other muscles included in the present study. Recent evidence suggests singing behavior is actively repressed in female zebra finches. Thus, I postulate these female-biased differences in syringeal NMJs might be a synaptic correlate of this suppression.

Results

I performed multi-color labeling and diffraction-limited confocal imaging of 27,620 NMJs across 3 types of muscles (syrinx, tongue, and *latissimus dorsi*) across sexes. This imaging enhances optical resolution via spatial filtering, using a pinhole aperture for selective light detection from a specific focal plane while rejecting out-of-focus light outside that plane. The result is sharp, high-contrast images of individual planes within a thin section, flattened together to obtain a crisp and comprehensive overview of all features of interest across the tissue. I labeled motor axons (immunohistochemistry for neurofilament), acetylcholine receptors (AChR, with α-bungarotoxin), and different muscle fiber types (IHC for fast myosin my32). For each NMJ, I classified the type of NMJ as *en plaque* (large, pretzel-like, and the dominant type at most mammalian and avian NMJs) or as *en grappe* (small, grape-like receptor staining, and almost non-existent in most mammalian and avian NMJs, see Methods). In addition, I measured their size, their organization (or lack thereof) into 'endplate' bands, the size and molecular type of their post-synaptic muscle fibers, and their innervation patterns by individual motor axons.

Major Synaptic Sex Differences of NMJs in Syrinx Muscles Only.

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In males, I observed numerous examples of NMJs that were large pretzel shaped, 'classical' *en plaque* type, (Fig. 1 A and B), found in the vast majority of striated muscles in mammals. In females, however, I found numerous examples of *en grappe* NMJs, "grape-like" or "beads on a string" (Fig. 1 C and D). Analyses over multiple samples (n= 3 samples, 11,349 NMJs) showed that male NMJs are almost exclusively *en plaque* (98.4% *en plaque*), while females show a roughly even mix of the two classes (51.3% *en plaque*, 48.7% *en grappe*, Fig. 1 E), one of the largest proportions of *en grappe* endings reported for any striated muscle in vertebrates (Smith and Rosenheimer 1982, Patterson and Pepperberg 1994, Lichtman and Wilkinson 1987). In addition, male *en plaque* NMJs are larger than female *en plaque* NMJs (Male: 850.0 μm^2, Female: 348.64 μm^2, $p < 1E$-26), which tracks with the larger muscle fiber size in males relative to females (Fig. 1 F, Male: 22.29 μm, Female: 13.41 μm, $p < 1E$-15). Muscle fiber size and NMJ area are not biased by hemisyrinx side or syringeal muscle fiber group (Supp. Fig. 1), nor is average NMJ size biased by the relative dorsal-ventral position within the syrinx (Supp. Fig. 2). Additionally, the relative shape of each NMJ class was consistent across sex, hemisyrinx, and syringeal muscle fiber group (Supp. Fig. 3). Importantly, these sex differences are not found in either of the control muscle groups, *lat* and tongue, where nearly all NMJs in both sexes were the *en plaque* class and with no sex differences in size (Supp. Fig. 4). Tongue muscles are innervated by axons from the same motor neuron pool as the syrinx (Bottjer and Arnold 1982, Nottebohm and Arnold 1976), suggesting that the sex differences in motor neuron synapses observed here are likely due to the muscle innervated rather than a general property of the innervating motor neurons. Finally, I note that NMJs across all muscles examined in both sexes were strikingly similar, except for the *female syrinx*, suggesting that sexual dimorphism in the vocal organ may be female-skewed, not particularly male.

en plaque NMJs organize into endplate bands, en grappe NMJs do not.

I next asked whether there were macroscale sex differences in the organization of NMJs. A common macro-organizational structure of NMJs is that synaptic sites are often clustered at similar locations along the length of muscle fibers, often resulting in the appearance of an 'endplate band'. I thus plotted the location of 714 NMJs across syrinx muscles of males and females. I found that *en plaque* NMJs clearly organize into a motor endplate band in the lower two-thirds of the muscle, regardless of sex (Fig. 2 A and B). However, female *en grappe* NMJs seemed to lack any such organization and were instead widely dispersed throughout the length of the muscle fibers (Fig. 2 C). I verified these observations by comparing the actual spatial distributions of male and female *en plaque* NMJs and female *en grappe* NMJs relative to randomly distributed junctions. I used a cumulative distribution function (CDF) to analyze the point pattern of NMJs relative to their distribution within the reference structure (the syrinx). I calculated the CDF of the distance between a typical point in the pattern and its nearest neighbor, compared this to the CDF calculated by a randomly generated point pattern, and calculated 95% confidence intervals over 500 iterations. I found that male and female *en plaque* NMJs differed significantly from randomized locations, i.e., they were more spatially confined, while the CDF of female *en grappe* positions was statistically indistinguishable from random locations (Fig. 2 D-F). *en grappe* NMJs in female syrinx are the only class and muscle analyzed here that fail to form a canonical motor endplate band, as both tongue *lat* show this classic macro-scale feature of innervation patterning. Together these findings indicate that *en grappe* endings show a similar lack of macro-organization as seen in other muscles with *en grappe* NMJs (Smith and Rosenheimer 1982, Jordan et al. 1989a, Fox et al. 2011), suggesting that NMJ type and not sex is the main determinant of macroscale distribution of NMJs.

Female motor neurons innervate all combinations of NMJ classes.

The existence of two qualitatively distinct types of synapses in female syringes naturally begs the question of whether distinct populations of motor neurons innervate each class. The local 'mixing' of *en grappe* and *en plaque junctions* in the female syrinx (Supp. Fig. 5) provides an opportunity to test whether individual motor neuron axons innervate either *en plaque* or *en grappe* NMJs exclusively or could innervate both NMJ classes. I started at NMJs of *en grappe* and *en plaque* NMJs and traced the neurofilament labeled axon to the first branch point. I then traced the branch point forward, when possible, and classified the type of NMJs innervated by each branch (n=156 starting axons). I found approximately equal incidences of the 3 potential types of innervation patterns, 50 branches (32.0%) innervated solely *en plaque* NMJs (Fig. 3 A, a), 53 (34.0%) innervated two *en grappe* NMJs (Fig. 3 B, b), and 53 (34.0%) innervated a *en grappe* and a *en plaque* NMJ in the endplate band where both types of NMJs could be found proximate to each other and consistent with previous reports in the mammalian extraocular (EOM) muscle (Smith and Rosenheimer 1982). Finally, unlike *en grappe* endings in mammalian EOM or snake transverse abdominal muscles (Smith and Rosenheimer 1982, Wilkinson and Lichtman 1985), I find no evidence of multiple innervation of *en grappe* NMJs by more than one motor axon in the adult syrinx. I conclude that the specialization of different NMJ types in females did not result in a corresponding specialization of motor neurons.

Differences in allometric scaling of synapse size and types of muscle fibers innervated by en plaque and en grappe NMJs.

NMJs generally scale allometrically with muscle fiber size: larger diameter muscles have larger synaptic innervation. With the prevalence of *en grappe* NMJs in the female syrinx, I wondered whether this scaling principle was true for all classes of NMJs. Thus, I divided our

NMJ area measures by class (*en plaque* vs *en grappe*) and compared synapse size to the diameter of the post-synaptic muscle fiber across samples. Allometric scaling principles were clear for all *en plaque* NMJs, across both sexes, even with the smaller average muscle fiber size in the female syrinx (Fig. 4 A, B; Male: R^2=0.644, Female: R^2=0.594, M vs F *en plaque* Z=1.49, $p = 0.14$). However, *en grappe* NMJs tend to be localized on the smallest diameter muscle fibers in the female syrinx and do not scale similarly to the *en plaque* NMJs with muscle fiber size (Fig. 4 C; R^2=0.082, M *en plaque* vs F *en grappe* Z=22.05, $p < 1E$-107; F *en plaque* vs F *en grappe* Z=18.21, $p < 1E$-73).

Given the stark differences between NMJ class and muscle fiber size, I asked whether these smaller *en grappe*-biased muscle fibers were on specific muscle fiber types. Muscle fiber type composition differs between male and female zebra finch syrinx, in the relative distributions of Fast and Superfast muscle fibers, as defined by myosin type. I processed a second series of each sample, to visualize to colocalization of NMJ class features with Fast vs Superfast fiber type (Fig. 5 A, B). I found that in the male syrinx, larger *en plaque* NMJs were found on larger Superfast fibers (Superfast NMJ = 933.69 μm^2, Fast NMJ = 634.42 μm^2, $p < 0.0001$), with a bias of Left larger than Right hemisyrinx (Left = 1041.2 μm^2, Right = 814.7 μm^2, $p < 0.01$), trends that were consistent in females but non-significant (Fig. 5 C) and consistent across muscle fiber groups (Supp. Fig. 6). Female *en grappe* NMJs were biased towards innervating Fast muscle fibers instead of Superfast muscle fibers (Fig. 5 D; 73.8% *en grappe* on Fast fibers, χ^2=16.1055 $p < 1E$-4), and even amongst Fast muscle fiber innervation, show little scaling with muscle fiber diameter (Supp. Fig. 6). Together, these findings indicate that there is a clear organizational bias towards *en grappe* NMJs appearing on the smallest and weakest muscle fibers in the female syrinx, indicating potential functional differences between fiber type, NMJ class, and behavioral output.

Discussion

Summary

I report that NMJs are qualitatively and quantitatively different in primarily the vocal organ of the zebra finch in adult males and females - a putative synaptic correlate for the sexually dimorphic behavior of singing seen in this species. I find little evidence of such differences in other muscles, even in muscles often associated with vocalization in other species, e.g., tongue (Patterson and Pepperberg 1994), but not in zebra finches (Bottjer and To 2012). I conclude that:

- **Evolutionary innovation can occur at the level of muscles.** Previous studies have demonstrated that evolutionary changes can often occur at the level of sense organs, e.g., eyes often evolve for nocturnal versus diurnal use (Hall et al. 2012). Our data suggest that similar changes can occur at the level of motor output, e.g., the innervation of individual muscle fibers. While there are likely sexually dimorphic differences 'upstream' of motor neuron innervation, in brain circuits, our data suggest that many of those brain differences may have evolved in the context of fundamentally different muscle innervation in males and females, providing a putative framework for better understanding those changes.

- **The female muscle is the 'special' case** Our data suggest that the sexual dimorphism in NMJs is in fact skewed female, and not particularly male - that is, the female syrinx appears to be the "special case". The NMJs of male syrinx muscles do not appear substantially different than NMJs of the *lat* or the tongue, in both males and females. Importantly, these muscles are used similarly by both sexes - the *lat* for flight, and the tongue for feeding behaviors. Thus, the sexual dimorphism is restricted to the muscles of vocal production, and most interestingly, these differences are particular to the female, and relate to the lack of behavior (song) output. Finally, since song is ancestral in songbirds of both sexes and more

recently lost in females of this species (Odom et al. 2014), I conclude that the sexual dimorphism I see primarily in females may have occurred to *suppress song production in female zebra finches*.

Limitations

Data was measured from n=3 birds of each sex in this experiment. While I sampled many thousands of NMJs across these samples, the small sample size of individuals may mean our data is underpowered. For instance, in our data on NMJ area, I see a trend in males of ventral syringeal muscles as larger than dorsal syringeal muscles. Since ventral muscles are more related to song production and dorsal muscles more related to inspiration/expiration (Wild 1997), this kind of bias solely in males would make functional sense. It is likely that the true state of potential differences between dorsal and ventral syringeal muscles could be uncovered with a greater sample size. Regardless, even a greater sample size would not alter the main conclusion of a sexual dimorphism in syrinx NMJ class that is female-skewed.

Data was measured from a single series of thin sections (series=3, 50μm section thickness) for all samples. This leads to two potential limitations. First, the endplate band analysis was able to be completed only for the ventral syringeal (VS) muscles, as samples were oriented with VS *en face* prior to sectioning, and these are orientations for published atlases that was necessary to differentiate muscle fiber groups. This orientation allowed for the clearest visualization of the VS muscle group, partly at the expense of single-section visibility of other entire muscle groups in a single section. Thus, I cannot say with statistical certainty that the lack of female *en grappe* endplate band organization is consistent across all muscle groups of the female syrinx. However, visual inspections and rough approximate reconstructions indicate this feature to be broadly consistent regardless of muscle group.

A second potential confound of thin sections is a clear positional bias in our axonal innervation pattern analysis, as I can only trace axons within the extent of the section z-thickness. It is not only possible but highly probable that axons travel outside of these volumes, and if I were able to efficaciously trace axons completely, the proportion of e.g., hybrid innervation patterns of both NMJ classes may differ from what I report. However, the occurrence of any number of axons innervating both classes means even if I were able to fully reconstruct axonal arbors with absolute certainty, this hybrid innervation pattern would still be present. Perhaps all axons in such an analysis would end up innervating both classes of NMJs, which would be an interesting outcome. Regardless, the occurrence of axons innervating both classes even in our thin samples is an interesting finding, and means there is not a specialized population of motoneurons solely innervating *en grappe* NMJs in the females.

Relevance to Prior Literature

Most of the prior literature detailing sexual dimorphism at NMJs show primarily quantitative differences in NMJs - the sex engaging in the behavior has larger NMJs of the same class - but not differences in kind, e.g., *en grappe* vs *en plaque*. Second, in other systems such as the mammalian extraocular muscle (EOM) where both NMJ classes have been found in the same sets of muscles, the proportion of *en grappe* NMJs is typically dwarfed by the classic *en plaque* class, at around 10% prevalence - strikingly lower than the 50% I report here. Data similar to our proportions of *en grappe* NMJs have been reported, for instance, in the garter snake (Wilkinson and Lichtman 1985). However, these *en grappe* NMJs are found on tonic fibers and are often multiply innervated by more than one motoneuron, features that make comparisons to our data tenuous. Third, the syrinx in both sexes is composed of Fast and Superfast (twitch) muscle fibers (Christensen et al. 2017), which in the vast majority of species are restricted to containing the

classic *en plaque* NMJ type. There is evidence from other clades, namely insects (Stocker et al. 2018, Wang et al. 2021) and teleost fishes (Bergman 1967, Nakajima 1969, Nishihara 1966) of *en grappe* NMJs found on these twitch-type fibers. However, though exact NMJ features may differ across these species or muscles, in each case they contain only the *en grappe* class of NMJs, not a mix of classes. Thus, to our knowledge, this is the first report of four key features of particular interest:

1. a sexual dimorphism in kind (class) of NMJ in the same muscle of any species

2. one of the highest proportions of *en grappe* in any muscle in vertebrates

3. a muscle composed solely of twitch-type fibers that shows a mixture of NMJ classes

4. a relationship between NMJ class and presence on Fast vs. Superfast fiber type

Additionally, I find in the female syrinx predominantly single innervation of *en grappe* NMJs. In other examples such as the mammalian EOM or the garter snake abdominal muscles, *en grappe* NMJs are multiply innervated, or contacted by multiple motoneuron axons (Lichtman et al. 1985, Fox et al. 2011). One possibility, for future investigation, is that *en grappe* NMJs in this system show signs of synaptic pruning that has been widely demonstrated for *en plaque* NMJs (Kasthuri and Lichtman 2003, Meirovitch et al. 2021).

Although the tongue is an important part of sound production in other vocalizing species such as humans, there is no evidence to suggest that the tongue is a necessary part of zebra finch vocalizations (Patterson and Pepperberg 1994, Bottjer and To 2012). The same is broadly true for other songbird species, with very rare evidence of the role of the tongue in vocal production. It may be used as a sound source dampener as it sits at the top of the vocal tract, though remains

somewhat independent of the lower vocal tract where the syrinx resides and is not a major player in the spectral features of song production. I see no sex differences in our data from the tongue, as both males and females have similar average NMJ size and are primarily the *en plaque* class. Our data cannot speak to the functional role of the tongue in vocal production of the zebra finch songbird; however, our data suggests that if there is such a role, it is likely not sexually dimorphic - and therefore would be related to vocalization broadly and not song specifically.

Methods

All procedures were conducted in accordance with the National Institute of Health guidelines for the care and use of animals for experimentation and were approved by the University of Chicago Institutional Animal Care and Use Committee.

Experimental Animals and Tissue Preparation.

All birds used in this study were bred at the University of Chicago, housed on a 12h:12h light:dark cycle, with seed and water provided *ad libitum*. All tissues were taken from adult birds, at least 120 days post-hatch. Three tissue samples were collected per bird – the syrinx, lingual muscles (tongue), and flight muscles (*latissimus dorsi*, "*lat*"). I assayed males and females, n=3 for each combination of sex and tissue type. Birds were collected from our animal facility and overdosed with isofluorane. Muscles were rapidly extracted and drop-fixed in 4% paraformaldehyde in 0.1 M PBS overnight at 4°C, then embedded in gelatin (8% in 0.1M PBS). Gelatin cubes were refixed in 4% paraformaldehyde for 2 hours at room temperature, then cryoprotected in 30% sucrose in 0.01 M PBS at 4until sinking. Gelatin cubes were nicked with a razor blade for *post hoc* orientation and sidedness identification, mounted in OCT media (Fisher) and cryo-sectioned at 40 *μm* thickness into a series of 3. Tissue was stored in PBS at 4°C until further processing.

Fluorescence Immunohistochemistry.

The protocol was performed on all sections from a single series, at room temperature unless otherwise noted. Tissue was rinsed in 0.01 M phosphate buffered saline (PBS) for 30 minutes, then blocked in 5% bovine serum albumin (BSA) for 1 hour. One series was incubated for 72 hours at 4°C in primary antibody solution: PBS supplemented with 0.3% Triton X-100 and 2% BSA (PBT+), including mouse anti-neurofilament (NEFM; 1:500, Thermo No.13-0700), rat anti-Substance P (1:1000, BD Biosciences No.556312). Sections were rinsed extensively in PBT+, then incubated in the secondary antibody solution for 2 hours: α-bungarotoxin (BTX; 1:100, Invitrogen No.A32728), goat anti-mouse Alexa Fluor 647 (1:500, Invitrogen No.A32728), and goat anti-rat Alexa Fluor 488 Plus (1:500, Thermo No.A48262). Sections were extensively rinsed in PBS, mounted onto Superfrost Plus slides (Fisher), and briefly dried before being coverslipped using ProLong GOLD Anti-Fade (Fisher No. P10144). A second series was processed following a similar protocol with few alterations: mouse anti-neurofilament was replaced with mouse anti-MY32 (1:100, Thermo No.M4276), and Wheat Germ Agglutinin (WGA; 1:250, Thermo No. W11261) was added to the secondary antibody solution. All other steps, solutions, incubation times, and temperatures were unchanged.

Imaging and Quantification.

To assess the morphology of muscle fibers and NMJs, I captured images using microscopes at the University of Chicago Light Microscopy Core. Images were captured on a Marianas Spinning Disk Confocal microscope (3i) with a 20x plan-neofluar 0.5 numerical aperture (NA) objective and a 100x alpha plan-fluar 1.45 NA objective. All samples were imaged with identical collection parameters. Z-stacks were captured at a step-size of $1\mu m$ for all tissues, and syrinx samples were captured as stitched mosaics of the entirety of tissue sections for efficacious

identification of muscle groups. Z-stacks were flattened using the two-dimensional maximum intensity projection algorithm in ImageJ (NIH) before analysis. Images were hand-annotated and quantified using ImageJ for measurement.

For our quantification of the syrinx samples, I focused only on the intrinsic syringeal muscles, or those with both attachment sites internal to the syrinx, organized into four major muscle groups. I describe these following the published 3D syrinx morpheme atlas:[52] the ventral syringeal muscle group (vS); the ventral tracheobronchial muscle group (vTB; deep and superficial ventral tracheobronchial muscles); the dorsal syringeal muscle group (dS; medial, lateral, and deep dorsal syringeal muscles); and the dorsal tracheobronchial muscle group (dTB; dorsal and short tracheobronchial muscles). Anatomical landmarks to identify the separate syrinx muscle groups are visible, and specific boundaries used to consistently quantify muscle groups were informed using this atlas. For the syrinx, I separated our quantification into right and left hemisyrinx, since there is known lateralization in both muscle size and syringeal function (Wade and Buhlman 2000, Wade et al. 2002). Tongue and *lat* analyses were not similarly separated, due to no known priors for lateralization in these muscles.

For all tissues, fiber size was calculated using the line tool in ImageJ, by measuring the diameter of individual muscle fibers nearby the visualized NMJs. NMJ area, circularity, and related measurements were calculated using the freedraw tool in ImageJ to individually outline the visualized NMJs by hand. NMJs were only quantified when it was readily apparent there was no superposition of multiple NMJs through the maximum intensity projections. NMJ class is easily discernible and was noted by the experimenter during measurements. For the analysis correlating fiber diameter to NMJ area, both were measured in the same manner as above. For the analysis of the proportional representation of NMJ class to muscle fiber type, quantification

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was performed separately for each subset of muscle fiber types within a muscle group. All output measures were first averaged across all measures within a single bird (and for syrinx, hemisyringeal muscle group), and these within-bird means were further used for statistical analyses.

Statistics.

Statistical tests were run with the anova function (analysis of variance; ANOVA) with Type III analysis in R (R version 3.5.1, stats package v 3.6.0) with $\alpha = 0.05$. For tongue and *lat*, these analyses were for main effect of sex; for syrinx, these analyses were for main effects of sex, hemisyrinx, muscle group, and all two-and three-level interactions. Parametric *post hoc* analyses were performed using the TukeyHSD function in the stats package. The same statistical tests were run for the second series analyzing the relationship between muscle fiber type and NMJ class. For the relationship of NMJ area to muscle fiber diameter, area measures were first log_2 transformed, then simple linear correlations output, with Fisher's z-transformation run for statistical comparison of correlation coefficients using the *cocor* package in R. Motor endplate band analyses were run using the 2D/3D spatial statistics plugin from the 3D ImageJ Suite, with the syringeal muscles as the reference structure and the binarized NMJs as the particles (point pattern) for dispersion analysis. Dispersion was assessed using the *F*-function to analyze the cumulative distribution function (CDF) of the distance between a typical position within the reference structure and its nearest point in the pattern, compared to the CDF if the points were randomly distributed throughout the reference structure. For the proportional representation of NMJ class to muscle fiber type, analyses were run using χ^2 tests for expected frequencies if there were no association between the variables.
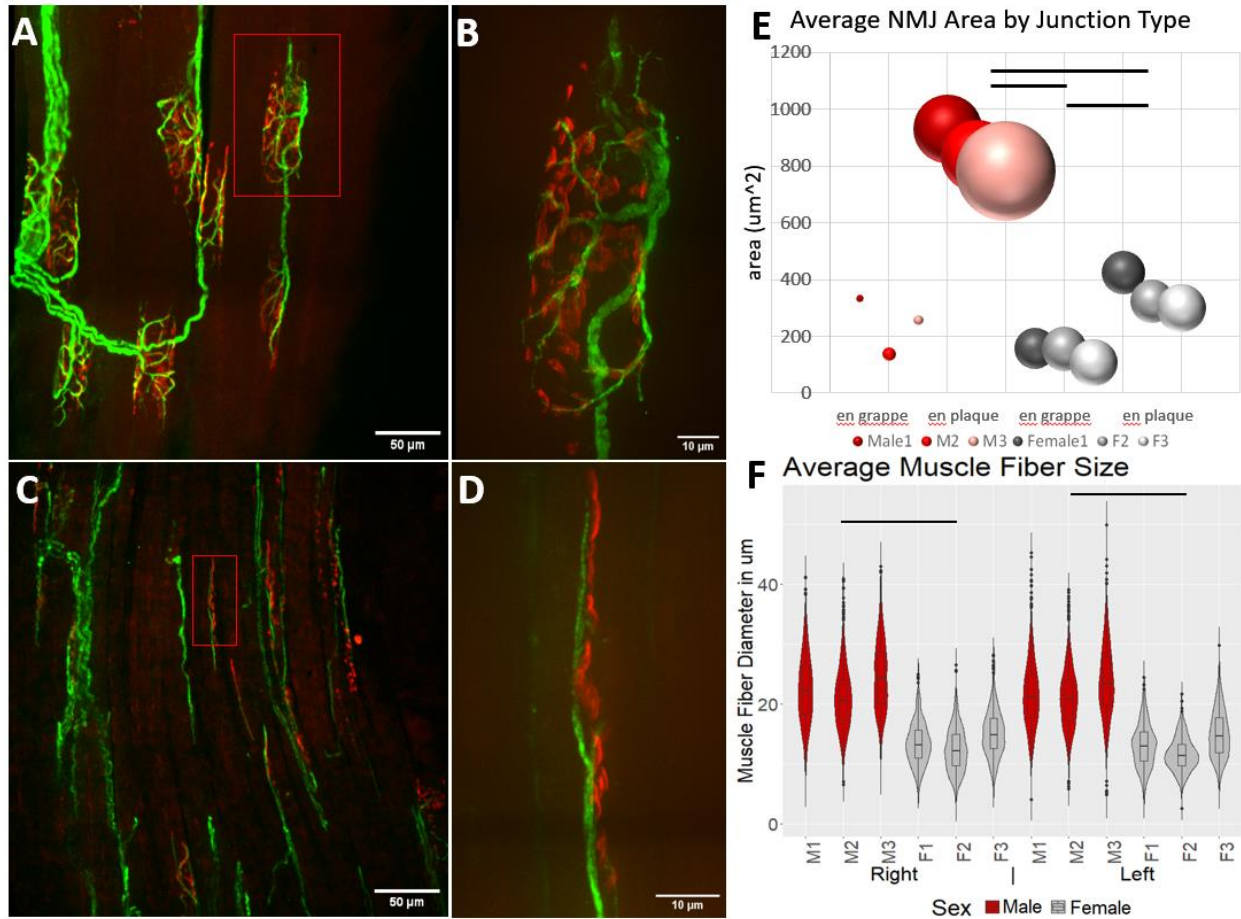
Figure 2-1: Qualitative and quantitative sex differences at individual Syrinx NMJs. A-D: Shown are maximum intensity images of male and female NMJs stained with neurofilament (axons, *green*) and a-bungarotoxin (synaptic receptors, *red*). A typical synapse from a male syrinx muscle is classical, latticelike *en plaque* NMJs (A, B) while many female NMJs are ribbon-like *en grappe* synapses (C, D; A and C 20x objective, B and D 100x objective. Red boxes in A and C are high magnification captures in B and D). Over multiple animals and 18,047 NMJ analyzed, 94.67 percent of male NMJs are *en plaque* while female NMJs show a more even distribution of 53.9 percent *en plaque* and 46.1 percent *en grappe* NMJs ($p < 1E$-16). *en plaque* NMJs were larger than *en grappe* overall (E, $p < 1E$-10) and males were larger than females for both *en plaque* junctions (E, $p < 1E$-26) and muscle fibers (F, $p < 1E$-15). These results are consistent across hemisyrinx (Left vs Right, F), syringeal muscle groups (Supp Fig 1), and dorsal-ventral position in the syrinx (Supp Fig 2). Bubble plots (E) show mean area within a subject on the x axis, with each bubble scaled in size to represent the number of measures being averaged. Violin plots show density distribution of muscle fiber sizes within a subject on the x axis, with box and whisker plots inset, and split into left and right hemisyrinx.
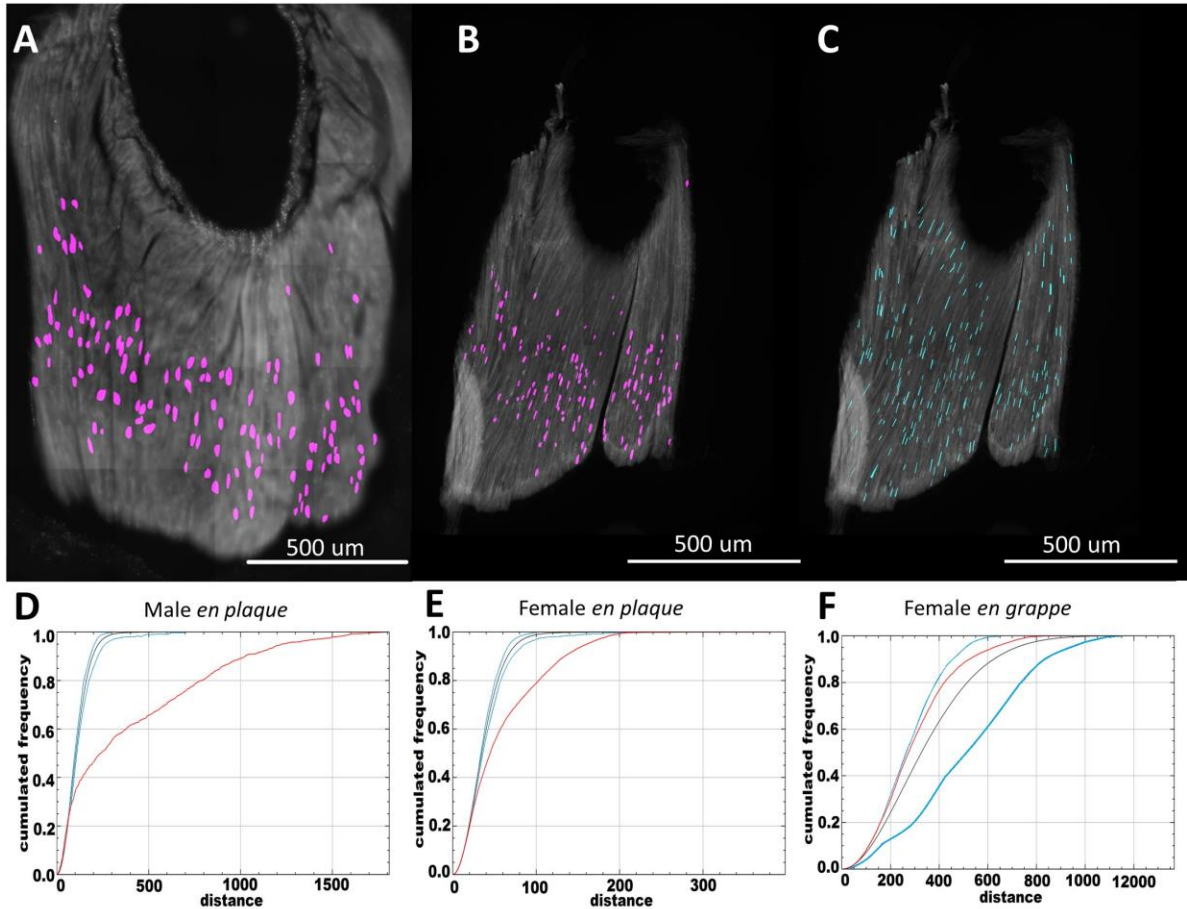
Figure 2-2: *En plaque* NMJs form classic endplate in bands in both sexes while female *en grappe* NMJs do not. A-C: Shown are maximum intensity projections of the ventral syringeal muscle in male (A) and female (B, C) syrinx. NMJs have been color-coded magenta (*en plaque*) or cyan (*en grappe*.) *En plaque*) NMJs organize into classic motor endplate bands in the lower 2/3 of the muscle in both sexes (A, B), while *en grappe* NMJs are distributed throughout the length of the muscle fibers in female syrinx (C). D-F. Cumulative distribution functions (CDF) comparing all distances between randomly distributed NMJs (black lines represent random samples and dashed lines represent the 95% confidence interval) to observed distances (red lines) for male *en plaque* (D), female *en plaque* (E), and female *en grappe* NMJS (F). The male and female *en plaque* observed distributions fall outside of the 95% confidence interval, indicating they are restricted into endplate bands, while the female *en grappe* distribution falls within the 95% confidence interval, indicating they are spatially random. See **Methods** for details.

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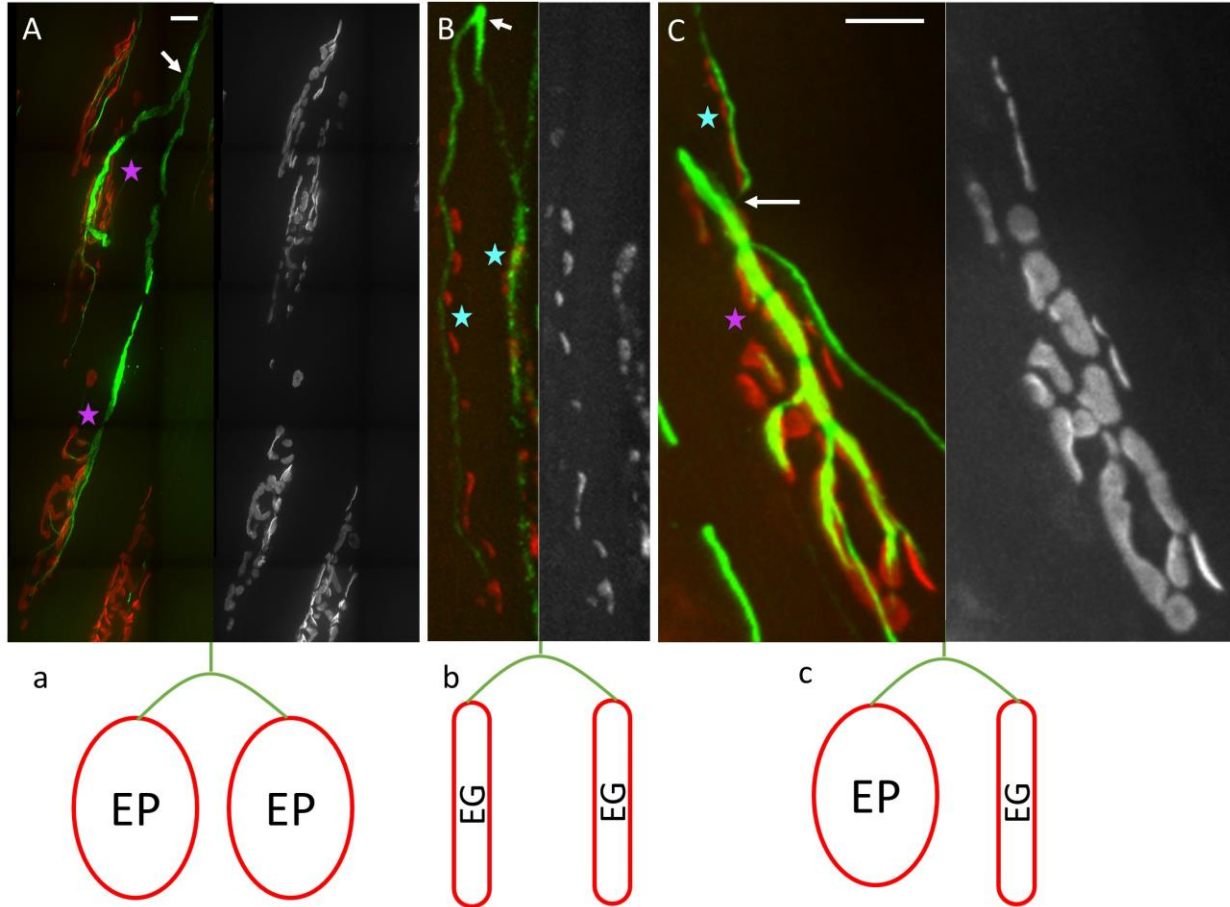
Figure 2-3: Motor neurons innervate both *en grappe* and *en plaque* NMJs. A-C: Shown are maximum intensity projection images of NMJs stained as in Fig. 1. a-c: cartoon diagrams of different general innervation patterns depicted in A-C. In female syrinx, we found every possible combination of axonal innervation pattern. Female axons may innervate only *en plaque* NMJs (A, a; 32.0%), only *en grappe* NMJs (B, b; 34.0%), or a combination of both NMJ types (C, c; 34.0%). Arrows point to motor axon branch points and asterisks denote NMJs of different types (magenta: *en plaque*; cyan: *en grappe*). Shown also in grayscale are only receptor stains to better appreciate NMJ type. Scale bar is 20 *μm* for each.
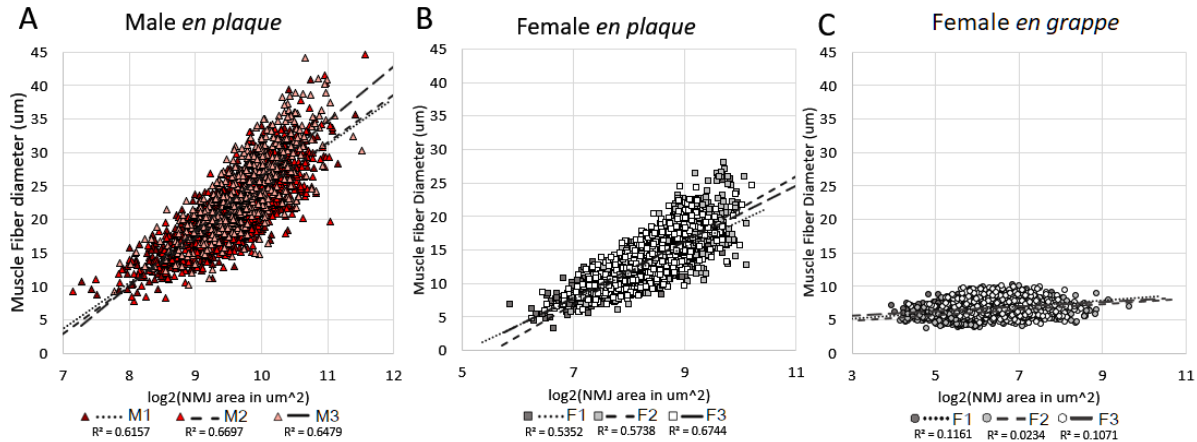
Figure 2-4: *en plaque* NMJ sizes scale with muscle fiber diameter, *en grappe* NMJs do not. Shown are 2D plots of NMJ area (log) versus muscle fiber diameter for (A) male *en plaque*, (B) female *en plaque* and (C) female *en grappe*. Lines are best linear fits for each of 3 samples, see figure for R2 values. *En plaque* NMJs show allometric scaling of area with muscle fiber size, regardless of sex (M vs F *en plaque* Z: 1.4874, p = 0.14), while *en grappe* NMJs occupy the smallest of the muscle fibers independent of synaptic area (M vs F *en grappe* Z: 22.0506, p < 1E-107; F *en plaque* vs F *en grappe* Z: 18.21113, p < 1E-73).
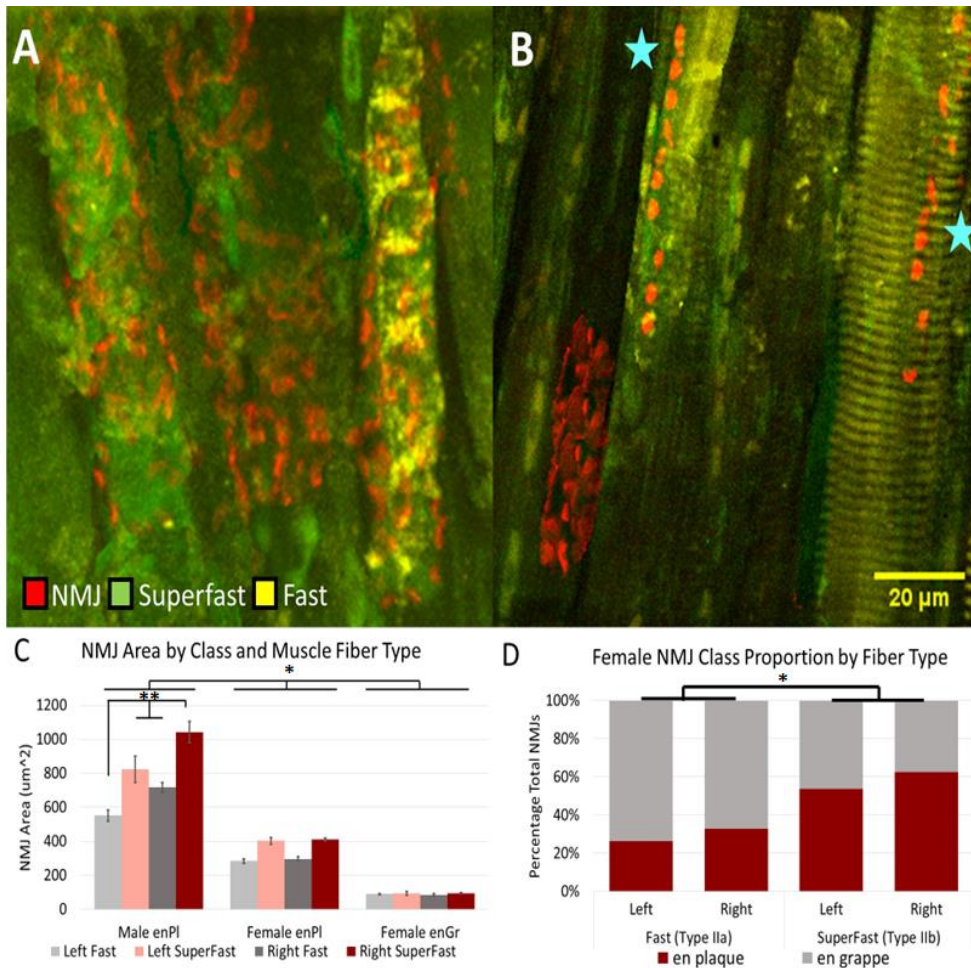
Figure 2-5: Fiber Type correlates with NMJ area for *en plaque* (both sexes), not *en grappe* (Females) – but *en grappe* NMJs are biased towards being present on the relatively smaller Fast muscle fibers. In Female syringes, Fast muscle fibers are biased towards having *en grappe* NMJs, while Superfast muscle fibers are more evenly distributed in their NMJs. A, B: NMJs in red (*en grappe* with cyan asterisks), superfast muscle fibers in green, fast muscle fibers in yellow. The male (A) primarily *en plaque* NMJs appear on both Fast and Superfast muscle fibers. Females (B) have many more Fast fibers than males, as previously reported. C: Muscles were first subset to muscle fiber type (Fast or Superfast), then the proportion of NMJ classes within muscle fiber types were quantified. ANOVA with TukeyHSD post hoc shows main effects of sex ($p < 1E$-13), side ($p < 0.01$), and fiber type ($p < 1E$-6), and a side*sex interaction effect for Males ($p < 1E$-4), not females (p=1). In Males, Left Superfast and Right Fast are not different ($p = 0.47$), but all other comparisons are different ($0.05 < p < 1E$-8). Female *en plaque* maintains the trend of SF>F but not R>L, and no comparisons pass significance. Female *en grappe* shows no significant differences. These are consistent across muscle group (Supp. Fig. 5). D: Females show a bias for *en grappe* NMJs to appear on the weaker Fast muscle fibers (*en plaque* 20.4%, *en grappe* 69.6%, χ^2: 16.1055, $p < 1E$-4), while NMJ class is more evenly split in Superfast muscle fibers (*en plaque* 58.0%, *en grappe* 42.0%). There is no left vs. right hemisyrinx lateralization. Male data was not similarly quantified due to the relative rarity of male *en grappe* NMJs identified.

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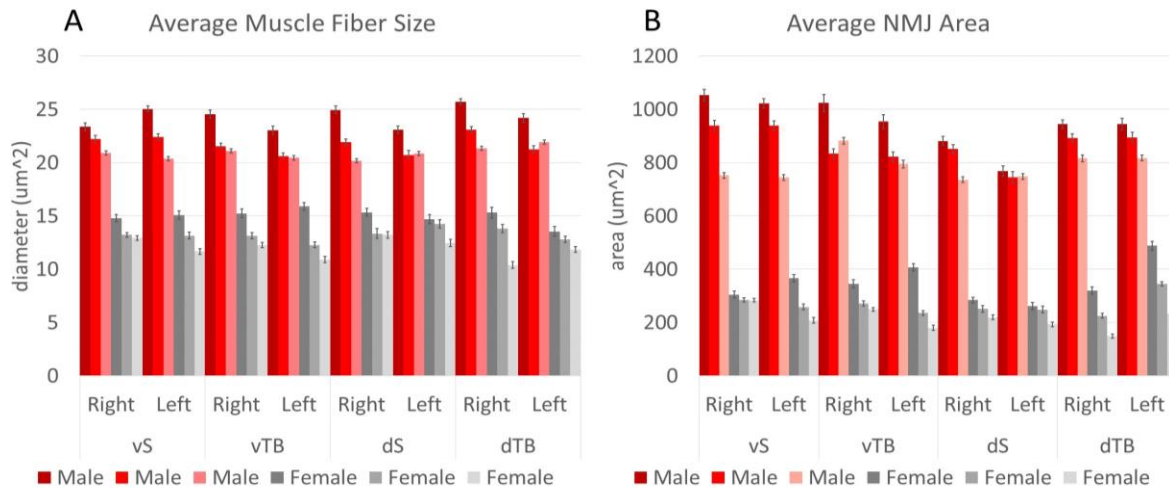
Figure 2-S1: **Muscle Fiber Size and NMJ Area Are Consistent Across Hemisyrinx and Muscle Fiber Group Within Sex**. A: Shown are average muscle fiber diameters for each individual male (red hues) and female (gray hues), split into individual muscle fiber group and hemisyrinx. Male muscle fibers are consistently larger than female muscle fibers, but there is no broad individual difference either within sex or across muscle fiber group or hemisyrinx. B: Shown are average neuromuscular junction areas for each individual male and female (same as A), again split into muscle fiber group and hemisyrinx. Similarly, male neuromuscular junctions are consistently larger than female, with no broad individual difference across muscle fiber group or hemisyrinx. Bars are means; errors are SEM. vS = ventral syringeal muscle group; vTB = ventral tracheobronchial muscle group; dS = dorsal syringeal muscle group; dTB = dorsal tracheobronchial muscle group.

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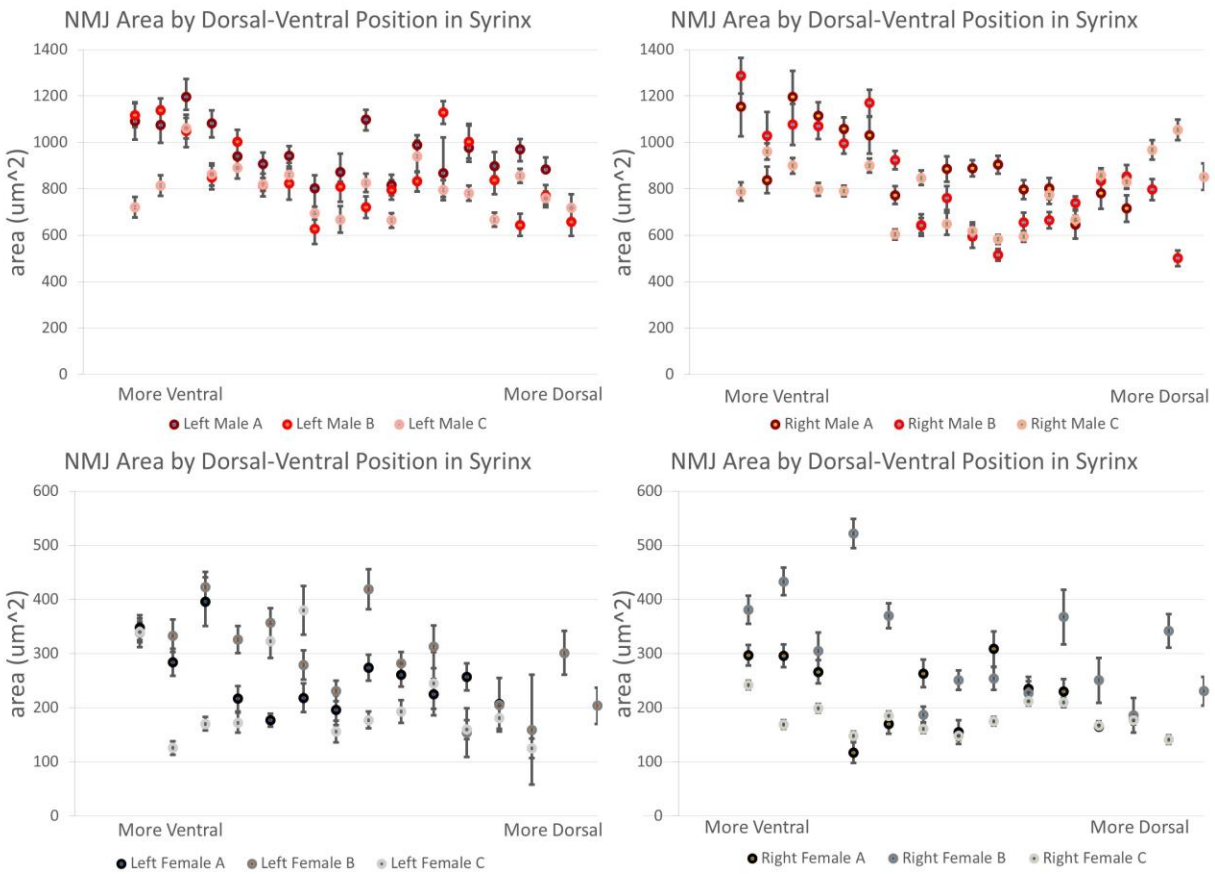
Figure 2-S2: NMJ Area is Consistent Across Dorsal-Ventral Extent of Syrinx for Both Sexes. Shown are average neuromuscular junction area for each relative slice position within the syrinx. Dots are mean, errors are SEM. Splitting measures into Left hemisyrinx (left column) or Right hemisyrinx (right column) for both males (top row) and females (bottom row) show little consistency in trend either between samples or across individuals, indicating our measurements are not broadly biased based on location within the syrinx.
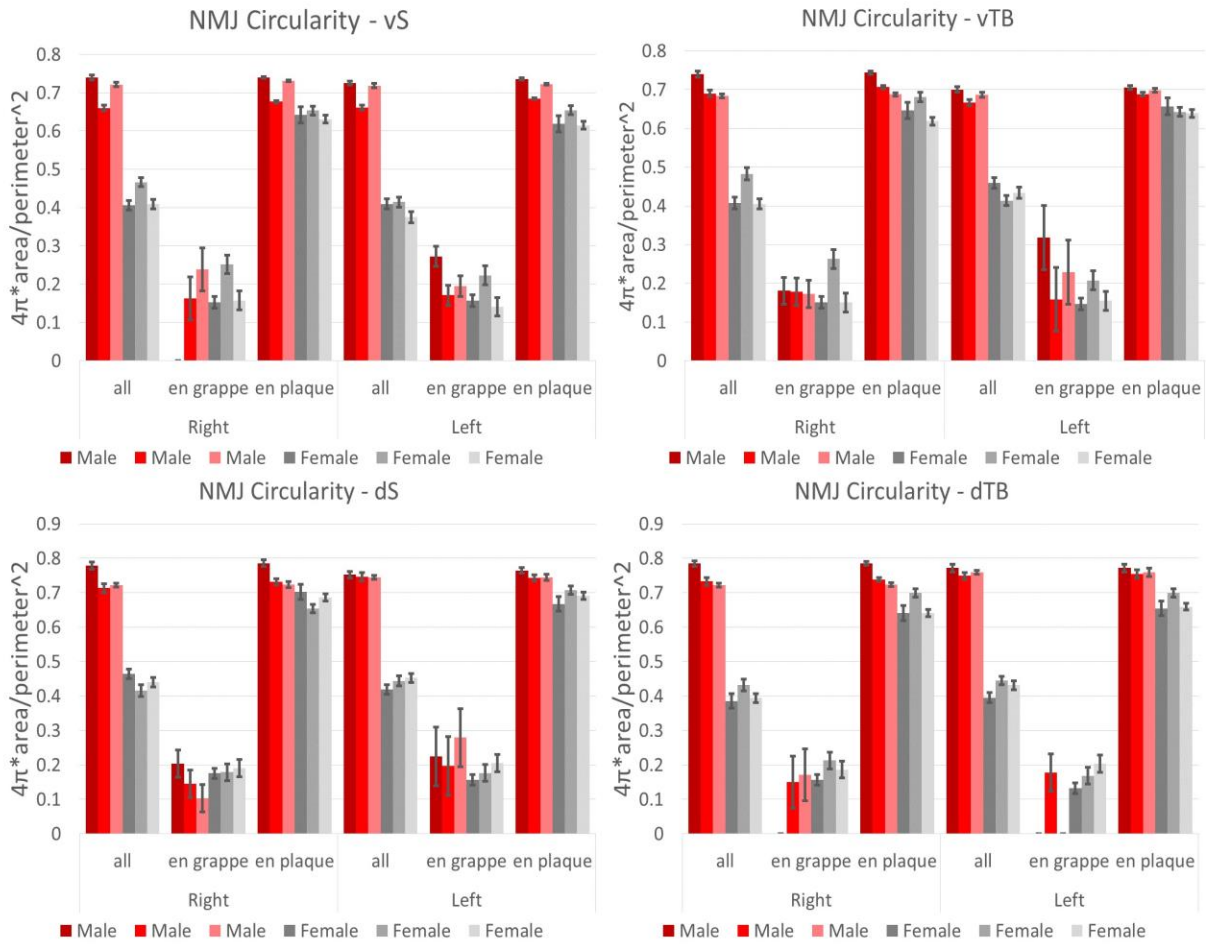
Figure 2-S3: Relative Shape of NMJs are Consistent Within Class and Between Sexes, Regardless of Muscle Fiber Group. Shown are numerical calculations of circularity, or relative shape of NMJs, with values closer to 1.0 being more circular. Each class has a similar relative shape, with *en plaque* NMJs being more circular, and *en grappe* NMJs being more ovoid. These shapes are consistent across muscle fiber group (plots), hemisyrinx (left vs right within each plot), and sex (bar hue). Bars are mean, errors are SEM.
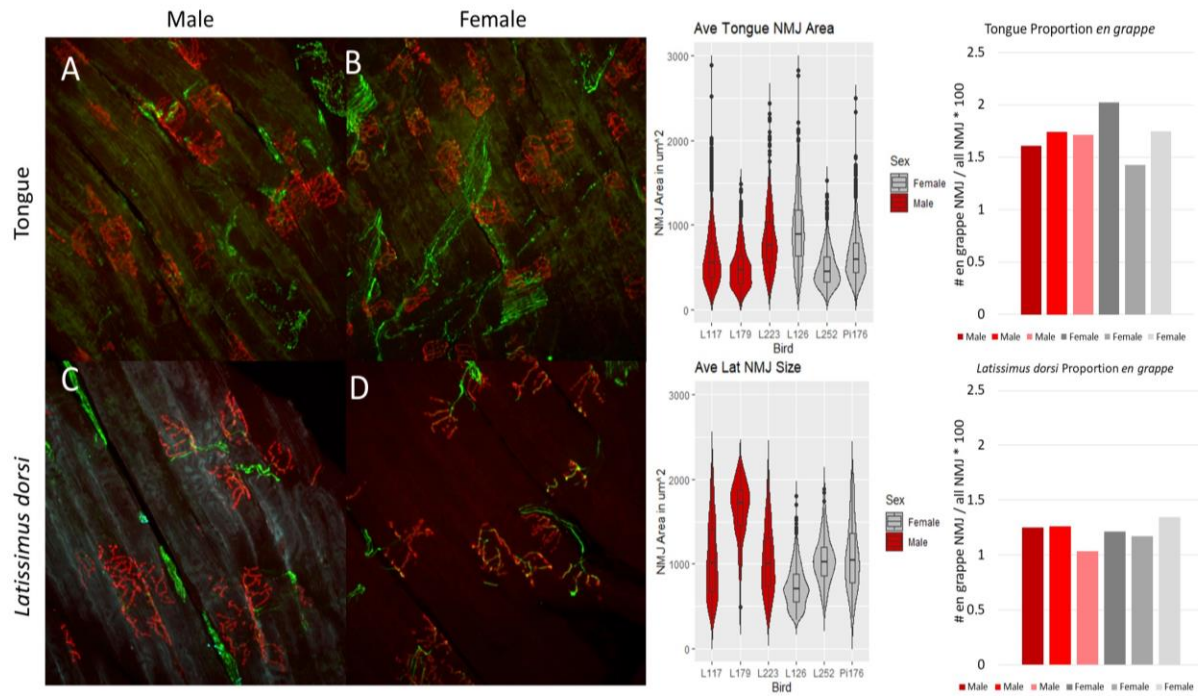
Figure 2-S4: Control Non-Vocal Muscles Show No Sex Differences in NMJ Size, With Predominantly *en plaque* NMJ Class. Shown are maximum intensity images taken with a 20x objective of male and female NMJs stained with neurofilament (axons, green) and a-bungarotoxin (synaptic receptors, red) same as in Figure 1. Both control muscles, tongue (A, B) and *lat* (C, D) show classical, lattice-like *en plaque* NMJs in both sexes. The average NMJ area is not sexually dimorphic (tongue: p=0.88; *lat*: p=0.92) and there are relatively few *en grappe* NMJs found in these muscles in both sexes (tongue: p=0.82; *lat*: p=0.55).
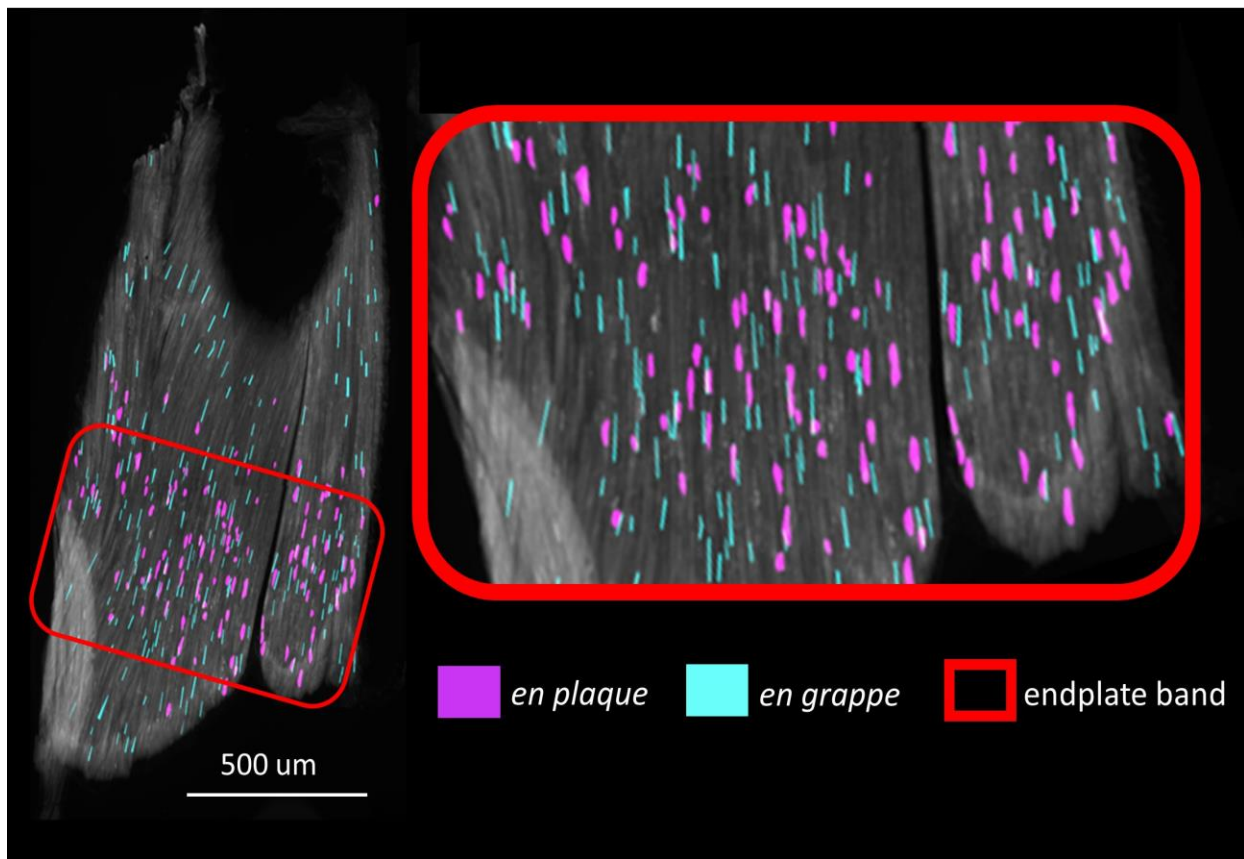
Figure 2-S5: Motor Endplate Band Region Shows Local Mixing of Both *en plaque* and *en grappe* NMJs. Left, an overlay of Figure 2B and 2C, showing an overview of a female syrinx slice marked with *en plaque* (magenta) and *en grappe* (cyan) NMJs. The red box outlines the endplate band region, which is inset on the right. Within this region, we see a local mixing of both types of NMJs, meaning there is no segregation between the two different classes within this region.
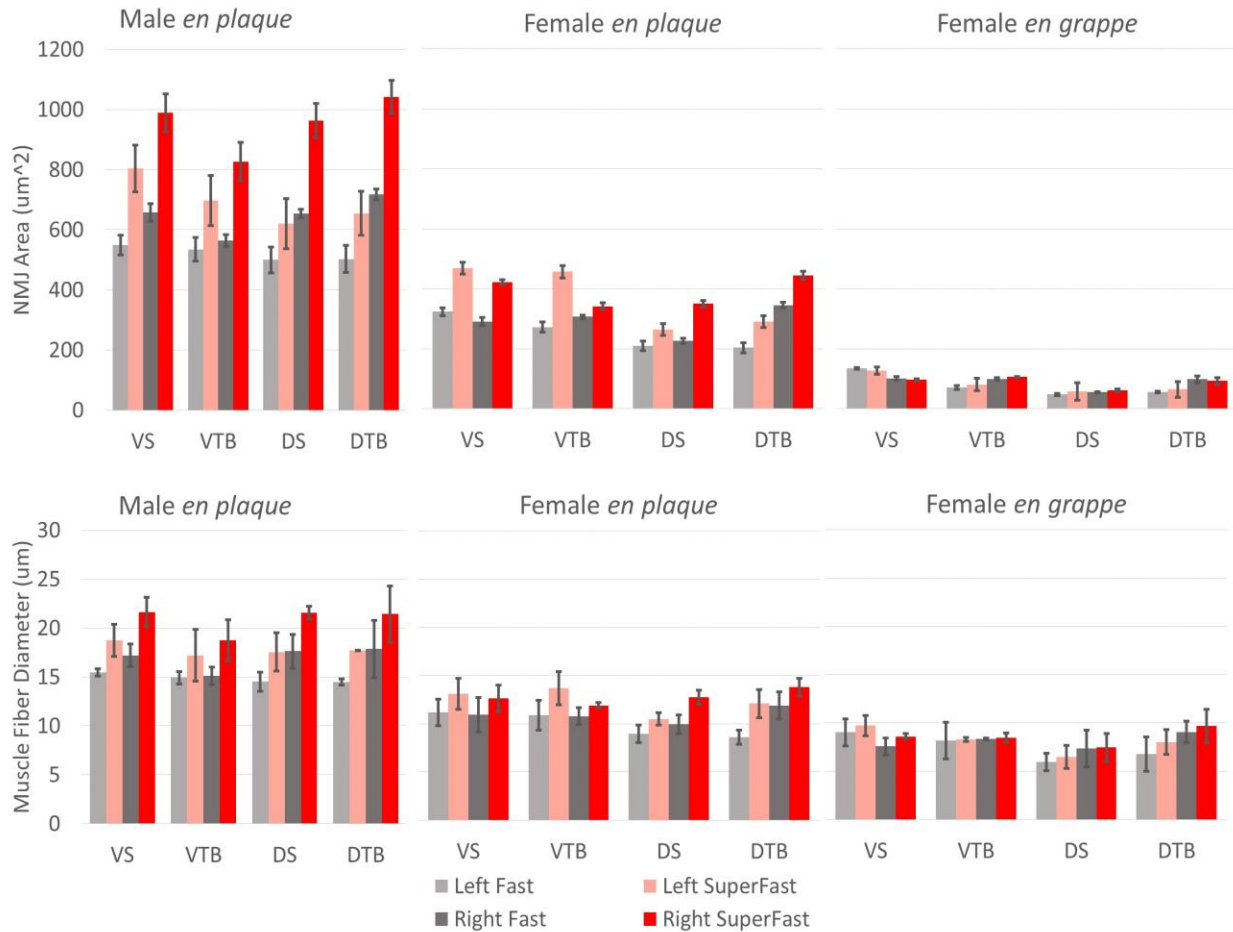
Figure 2-S6: Trend Towards Larger *en plaque* NMJs on Larger Superfast Muscle Fibers is Consistent Across Muscle Fiber Groups. Shown are average NMJ area (top row) and muscle fiber diameter (bottom row) for each NMJ type (columns). The trend of Superfast NMJs and fibers (red hues) being larger than their Fast counterparts (gray hues) for *en plaque* NMJs is consistent across muscle fiber group and hemisyrinx, while *en grappe* NMJs show no differences across subset measures.

Chapter III: Syrinx Synaptic Development Follows Two Divergent and Sex-Specific Trajectories

Introduction

Adult male and female bodies are different, yet early in development, these specializations have often not yet emerged. Adult specializations typically arise over development from multiple complex processes - genetic encoding, experience, and their interactions. Thus, there are multiple levels of regulation that may lead to adult sexual dimorphisms. One possibility is that, early in development, both sexes are highly similar or sex-neutral, after which one sex develops a specialization, and the other sex fails to do so. This would imply that the "non-specialized" sex in adulthood would be indistinguishable from either sex early in development - or, in other words, undeveloped or underdeveloped. Competing theories argue that, early in development, either sex has the *potential* to develop a specialization, and their adult phenotypic differences represent the outcome of two active developmental processes, in one sex promoting that development, and in the other suppressing that development and promoting an alternative developmental program and behavioral outcome.

Zebra finch vocal behavior provides an excellent opportunity to address these theories. Zebra finch vocal behavior is sexually dimorphic in adulthood - males sing complex learned songs, while females do not. Male song is learned during early post-hatch development, through a confluence of multiple overlapping learning programs - memorizing songs, producing immature songs, and then fine-tuning by comparing motor outputs to auditory feedback and song memories (Figure 1). The development of neural systems that support this behavioral dimorphism is well

defined (see Chapter 1), and the consensus is that nodes and circuitry of the male brain song system grow larger and more complex over development in support of song, while in the female, they atrophy.

Whether the same ontogeny extends to the syrinx is unknown. The muscles of the syrinx are present by embryonic day (E) 10, and in early post-hatch life, males and females have similar syringes by gross weight. Sex differences in size do not emerge until P20, near the onset of the developmental song learning phase (Godsave et al. 2002). This is also around the age that sex differences in muscle fiber size are first found (Lohmann 1997). Sex differences in the proportion of superfast muscle fibers likely emerge around or after the age males start producing immature songs (Mead et al. 2017), though the exact timing of this dimorphism is unknown, due to a lack of female developmental data. Similarly, how synaptic features develop in either male or female syringes is wholly unknown.

The results I observed in the syrinx of adult males and females in Chapter 2 demonstrate that the female syrinx is the special case among all muscles measured. This opens the question of how this adulthood sexual dimorphism arises. One possibility is that male and female zebra finches are hatched with sexually dimorphic synaptic arrangements in the syrinx. A more likely possibility is that, early in development, the synaptic morphology of male and female syringes is not sexually dimorphic, and those differences emerge across development. This leads to two broad predictions: either one sex further develops while the other sex fails to develop, or the two sexes diverge and each follows their own specific developmental program. Prior reports indicate that gross features of the syrinx are similar between males and females until P20, after which the male syrinx continues to grow into adulthood, while the female syrinx does not (Godsave et al. 2002). However, how syringeal synapses change over development is unknown. When and how these sex

differences emerge would inform as to whether the unique synaptic arrangement of the adult female syrinx, and the potential of those features for female song suppression, is supported by female-specific syringeal synaptic development, or lack of development therein.

Results

Summary

I performed multi-color labeling and diffraction-limited confocal imaging across sexes at 4 different ages (Figure 1): early life at post-hatch day (P) 10; near the onset of developmental song learning (P30); near the onset of immature song-like vocalizations (P50); and near the onset of crystallization of song learning (P70), after which songs become highly stereotyped. I labeled motor axons (immunohistochemistry for neurofilament), acetylcholine receptors (AChR, with α-bungarotoxin), different muscle fiber types (IHC for fast myosin my32), and muscle fiber boundaries (wheat germ agglutinin). For each NMJ, I classified the type of NMJ as *en plaque* or as *en grappe* based on morphology. In addition, I measured their size, their organization (or lack thereof) into 'endplate' bands, and the size and the type of their post-synaptic muscle fibers.

I found 4 main results from my analyses, summarized here and further explained in depth in following sections:

1. Males and females in early life are similar at all levels, e.g., proportion and size of NMJ classes and muscle fibers, contrasting the adult phenotype shown in Chapter 1

2. Development of *en grappe* NMJs, in particular, is sexually dimorphic at all levels, in distribution, relative proportion, and size

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3. Development of *en plaque* NMJs is sexually dimorphic in size and proportion, but not in distributional rearrangements

4. Increases in muscle fiber size and superfast fiber proportion emerge by P50, but are not substantially sexually dimorphic until P70

Syrinx Synaptic Organization is Sexually Monomorphic Early in Life.

At P10, I observed male and female syringes at the level of NMJs are indistinguishable from each other. These NMJs are similar to the adult *en plaque* and *en grappe* phenotypes, though smaller and, for the *en plaque*, more "plaque-like" than "pretzel-like" as found in their adult counterparts (Figure 2). Both males and females at P10 have roughly equitable proportions of these two NMJ classes (Figure 3; Males = 51.8% *en plaque*, 48.2% *en grappe*, p=0.26; Females = 50.6% *en plaque*; 49.4% *en grappe*, p=0.77), with no sexual dimorphism in size (*en plaque* = 277.4 μm^2 Male, 260.1 μm^2 Female, p=0.19; *en grappe* = 179.3 μm^2 Male, 195.3 μm^2 Female, p=0.85). Additionally, there is no sexual dimorphism at this age in muscle fiber size (Figure 4; Male = 11.44 μm, Female = 11.76 μm, p=0.41) or muscle fiber type (Males = 96.9% fast fibers, Females = 97.1% fast fibers, p=0.9). Finally, I note that both classes of NMJ may be found anywhere throughout the length of the syrinx muscles, as at this age there does not appear to be any centralized motor endplate band in either sex (Figure 2).

At P30, when male syringes become larger than females (Godsave et al. 2002), I continued to observe a lack of sexual dimorphism in synaptic organization. Both males and females at P30 are equitable in their proportions of *en plaque* and *en grappe* NMJs (Figure 3; Males = 50.9% *en plaque*, 49.1% *en grappe*, p=0.53; Females = 52.0% *en plaque*, 48.0% *en grappe*, p=0.3), with no sexual dimorphism in size (Figure 3; *en plaque* = 405.62 μm^2 Male, 376.68 μm^2 Female, p=0.19;

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en grappe = 159.2 µm^2 Male, 165.9 µm^2 Female, p=0.85). Additionally, there is no sexual dimorphism at this age in muscle fiber size (Figure 4; Male = 13.82 µm, Female = 14.45 µm, p=0.19) or muscle fiber type (Males = 94.8% fast fibers, Females = 95.8% fast fibers, p=0.27). Finally, I note that while both classes of NMJ may be found anywhere throughout the length of the syrinx muscles, there does appear to be a rudimentary motor endplate band beginning to form for *en plaque* NMJs in both sexes (Figure 2).

Thus, there is little difference in the development of *en plaque* NMJ across males or females. At P10 and P30, there is no sex difference in proportion or size of *en plaque* NMJs, and they are not tightly organized into a motor endplate band. By P50, both males and females have begun losing *en plaque* NMJs outside of the MEB region, i.e., have begun to form an *en plaque* MEB (Figure 2). By P70, this loss of extra-MEB *en plaque* NMJs is nearly complete in both males and females.

The major difference was how they changed in size. Male *en plaque* NMJ sizes increase at every age (Figure 3; P10 = 277.4 µm^2, P30 = 405.6 2 µm^2, P50 = 570.95 µm^2, P70 = 806.77 µm^2, p=0.026). This size at P70 is nearing the average size of these NMJs of adult males (850.0 µm^2; see Chapter 2). Female *en plaque* NMJs, in contrast, reach their adult size by P50 (P10 = 260.1 µm^2, P30 = 376.68 µm^2, P50 = 362.33 µm^2, P70 = 371.43 µm^2, p=0.04). Thus, after P50, changes in female *en plaque* NMJs seem restricted to spatial reorganization while maintaining similar sizes into adulthood (adult female = 348.64 µm^2; see Chapter 2).

Developmental Reorganization of en grappe NMJs is Sexually Dimorphic.

En grappe NMJs are sexually dimorphic in their developmental rearrangements. Early in life, *en grappe* NMJs may be found anywhere throughout the length of the muscles in males and females, and are similar in size between males and females at P30 (Male = 159.2 µm^2, Female = 165.9 µm^2,

p=0.85). By P50, male *en grappe* NMJs have become smaller in size (103.94 μm^2, p=0.02). In contrast, at P50 the female *en grappe* NMJs have become moderately larger (184.89 μm^2, p=0.04). However, at this age the relative proportions of the two classes are not sexually dimorphic (Male: 50.2% *en plaque*, 49.8% *en grappe*; Female: 47.8% *en plaque*, 52.2% *en grappe*, p=0.61). In females at P70, the *en grappe* NMJs have become larger still than at P50 (229.98 μm^2, p=0.002), and are particularly larger than those in the males at P70 (70.9 μm^2, p<1E-4). However, the proportion of NMJ classes did not change (52.3% *en plaque*, 47.7% *en grappe*, p=0.39), and is similar to the proportions found in adult females (see Chapter 2). In contrast, by P70, males have lost the vast majority of their *en grappe* NMJs (72.4% *en plaque*, 27.6% *en grappe*, p<1E-3), though there are still a number of remnants visible, most apparent in the extra-MEB region and smaller than at P50 (, P50 = 103.94 9 μm^2, P70 = 70.9 μm^2, p=0.03). In addition, the male P70 proportion of *en grappe* NMJs is higher than in adult males (see Chapter 2), implying there is further reduction of *en grappe* NMJs between P70 and adulthood in males alone.

Male Bias Towards Larger Superfast Muscle Fibers Emerges Late in Development.

I next asked whether developmental shifts in the proportion of fast vs superfast muscle fibers was sexually dimorphic, and how this divergence might support vocal production differences. Contrary to the development of NMJs in the syrinx, I find instead a male bias in muscle fiber type development. Early in life, I find both male and female syringes are composed solely of fast muscle fiber type, with rare occurrences of superfast muscle fibers, at either P10 or P30. By P50, there is an increase in the proportions of superfast fibers in males (P30 = 5.2%, P50 = 15.3%, p=0.012), and a similar but more moderate change in age-matched females (P30 = 4.2%, P50 = 9.4%, p=0.038). By P70, the superfast proportion is larger than P50 in both sexes, but the magnitude of increase in greater in males. Male muscle fiber types have shifted to predominantly the superfast

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type (81.6% Superfast, 18.4% Fast), and while females still predominantly have the fast muscle fiber type (24.2% Superfast; 75.8% Fast), it has further increased relative to P50 (p<1E-4). Finally, I note that while average muscle fiber size increases between P10 and P30 in both males and females, there is no difference between sexes at P30 (Male = 13.82 µm, Female = 14.45 µm, p=0.47) or P50 (Male = 14.48 µm, Female = 15.50 µm, p=0.31), but in males there is an increase by P70 (24.17 µm, p<1E-4). Thus, male syrinx muscle fiber size and type appear not to reach adult-like levels until P70, while in females, this occurs for muscle fiber size by P30, and muscle fiber type by P70.

Discussion

In the zebra finch brain, developmental changes in the song system are sexually dimorphic, with those nodes and circuits growing larger and more complex in males in support of song, and atrophying in females. Whether this ontogeny broadly extended to the peripheral portions of the song system, namely the syrinx, was unknown. I show instead that males and females have distinct developmental programs that lead to their adult phenotype in the syrinx - not that the female syrinx fails to develop or atrophies relative to the male, but instead undergoes growth and refinement of synaptic and muscular components that might instead support song suppression.

The adult sexually dimorphic phenotype reported in Chapter 2 is ultimately realized over these two divergent developmental programs. I find, in particular, that this sexually dimorphic development is most apparent in the development of *en grappe* NMJs, and particularly those in the portion of the syrinx outside of the canonical MEB region. In males, these NMJs grow smaller and more fractured, ultimately likely to be pruned away by adulthood, while in females, they become the sole extra-MEB NMJ, and grow larger with age. I conclude from these data that the adult female phenotype observed in Chapter 2 is actively developed - that is, not that the adult

female is simply an undeveloped or underdeveloped male, but that there is a female-specific developmental program at the level of syringeal NMJ anatomy. I also conclude that female song suppression in this species might be supported by enlargement of *en grappe* NMJs across development, particularly in the region outside the MEB, while their non-song vocal ability is likely supported by refinement of *en plaque* NMJs into a MEB, similar to what I observe in males. However, little is known about potential developmental vocal changes in females, and further investigation would be necessary to make a stronger claim therein. Finally, my results imply that early male song production might rely on, or drive, the emergence of superfast muscle fibers, while crystallization processes might rely, in part, on both the continued growth or transition towards predominantly superfast fibers, as well as the removal of those *en grappe* NMJs from outside of the MEB region.

The male synaptic and muscular refinements across development that support song production raise a few outstanding questions, both for these birds specifically and NMJ systems broadly. First, males show a moderate increase in superfast fiber percentage between P30 and P50, and a further substantial increase by P70. This would imply that superfast fibers are not wholly necessary for immature song production, but are related to more stereotyped near-adult song behavior. However, the directionality of this relationship, in both this system and other well-described NMJ systems, is unclear. Components of these systems (NMJ size, muscle fiber size, and superfast muscle fiber proportion) are tightly correlated and tied to use, e.g., more use of a muscle group tends to increase all these components, but with no strong evidence that one or some precede the others (Balice-Gordon et al. 1991a; Balice-Gordon et al. 1991b). Therefore, an argument could be made that immature vocalizations around P50 are variable, in part, due to the relative lack of superfast muscle fibers in the syrinx. Thus, continued practice between P50 and

P70 could lead to the conversion of fast fibers into superfast fibers, and then gradual stereotypy of song production. An equally valid argument could be made that this period of immature song practice first aims to stereotype the NMJs and behavior, after which continued production of a less variable set of muscular motor movements then strengthens those muscle fibers, thus necessitating emergence of superfast muscle fibers to maintain high efficacy of behavioral reproduction. Further investigation of which of these components might be the driving factor in male development is necessary, and could perhaps be uncovered via targeted knockdown of the superfast myosin gene in development, or additional developmental ages for collection both within and after development.

Similarly, the female synaptic and muscular refinements across development that might support song suppression raise a few outstanding questions. First, the relative location of NMJs across muscle fibers, i.e., formed into a MEB or spatially distributed, are well-documented in other models in direct relationship to muscle function (Fox et al. 2011; Purves and Lichtman 1985; Mech et al. 2020). Spatially distributed NMJs are a classic feature of immature NMJ systems, and part of the reason why juvenile muscular motor movements are typically more variable or weak than in adulthood. However, this conclusion is confounded by the fact that individual NMJs in these models also grow larger and stronger over development and repeated muscle use. I report *en grappe* NMJs in the female that also grow moderately larger over development, but do not organize into an MEB. Thus, one might conclude these female *en grappe* NMJs are also growing individually stronger over development, though as of yet there is no functional electrophysiological data on this NMJ class in this model. It is tempting to think that larger and stronger *en grappe* NMJs that are highly variable in distribution might provide a sort of destructive interference to the complex muscle movements necessary for song production, thus supporting

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song suppression in the female. Further functional data on the relative synaptic strengths of these two NMJ classes in this species is needed.

Second, adult females vocalize as much as adult males, having the same vocal repertoire except for song (Elie et al. 2011; Zann 2006). These non-song vocalizations are still highly stereotyped and able to be modified in a context-dependent manner in adulthood (Benichov et al. 2016). Thus, the female developmental reorganization of *en plaque* NMJs into a MEB would imply that these synapses might be important for non-song vocal production in general. However, there is a lack of study on female vocal development, and it is unknown whether female non-song vocalizations become stereotyped or "crystallized" like male songs across development, or whether they emerge in a stereotyped state. There is evidence that, early in life, both males and females primarily produce similar begging calls, which are not produced by adults (Elie et al. 2016). When and how non-song vocalizations emerge in females, and how they might change over development, would be necessary to make functional claims about female *en plaque* NMJ reorganization over development in relation to female vocal behavior.

Interestingly, I see a number of NMJs that appear intermediate between *en plaque* and *en grappe* at these ages, i.e., a typical *en plaque* NMJ with an *en grappe* extension, similar to an antenna. This could imply that, similar to muscle fiber types, NMJs may transition between *en plaque* and *en grappe* over development. However, the direction of that transition, and the ultimate outcome, is unclear. The developmental shift in proportions of *en plaque* and *en grappe* NMJs in males would imply that this transition is from *en grappe* to *en plaque*, though this opens the question of why there are this intermediate class NMJs in the females, whose proportion of NMJ classes does not significantly change over development. One possibility is that this intermediate class might be labile or transitory in either direction, from *en grappe* to *en plaque* or vice versa,

and that their presence in both sexes might indicate a central role not to shifting proportions of either class of NMJ across development, but of shifting regional distributions of these classes. Males across development "lose" *en plaque* NMJs first outside of the MEB region, by P70 only having small *en grappe* NMJs in this region; Females similarly "lose" *en plaque* NMJs outside the MEB, but by P70 having solely *en grappe* NMJs in this region. What I describe as a loss, might instead be a shift of individual NMJs between the classes: in males, from *en plaque* to *en grappe* before fully pruning away by adulthood; and in females, from *en plaque* to *en grappe* before enlargement or further refinement. A more rigorous assessment of the relative proportions of these NMJ classes within vs outside of the MEB across development would help determine whether this prediction holds true. More work broadly on the developmental flexibility of these NMJs would be required to elucidate these types of possible transitions.

We don't yet know the actual mechanisms by which these developmental changes in NMJ organization happen, but it is likely to involve processes of synaptic pruning. The classic model is the pruning of supernumerary connections of motor neurons at *en plaque* NMJs in rodents. Early in life, each *en plaque* NMJ is innervated by many motoneuron axons, which then refine over development to a single innervating axon, as the other axons are pruned away from that NMJ (Kasthuri and Lichtman 2003; Meirovitch et al. 2021). In those systems, this is a process driven by neuronal activity that parallels the maturation of behavior, and is thought to ultimately lead to the lattice-like internal structure of *en plaque* NMJs - representing the innervation sites of the "winning" axon and further refined, and the functional consequence of this plaque-to-pretzel transition of normal *en plaque* NMJ development. The transition from multiple to single innervation does not broadly appear to correlate with maturation of vocal behavior in the zebra finch model.

However, I have identified at least two different kinds of pruning that may correlate with behavioral maturation in our model. First, across male development, NMJs outside of the canonical MEB get smaller and more fractured with age, ultimately being largely absent by adulthood. This implies that pruning in this region is not necessarily only on the presynaptic side, but also on the postsynaptic side. Further detailed analyses of these processes would be useful to uncover whether the motoneurons have been pruned away first, and the lack of innervation predates the reduction in NMJ size, or vice versa. Second, early in development, many *en grappe* NMJs in both sexes have a morphology more like a straight and filled line, not grape-like or beads-on-a-string as I see in adults. This implies that there is active synaptic refinement of *en grappe* NMJs over development, pruning away of some ACh receptors on the postsynaptic side, and therefore potential pruning of supernumerary connections of motor neurons on the presynaptic side. This kind of development of *en grappe* NMJs has not previously been described in vertebrate models, likely because in models where *en grappe* NMJs are most well-studied, they are multiply innervated in adulthood, not singly innervated as I report here. Whether the same kind of supernumerary pruning occurs at *en grappe* NMJs in this model, and the exact processes that support this development, requires further study, and would be additionally informative to understanding the ultimate functional role of these synapses in vocal behavior.

Methods

All procedures were conducted in accordance with the National Institute of Health guidelines for the care and use of animals for experimentation and were approved by the University of Chicago Institutional Animal Care and Use Committee.

Experimental Animals and Tissue Preparation.

All birds used in this study were bred at the University of Chicago, housed on a 14h:10h light:dark cycle, with seed and water provided *ad libitum*. All tissues were taken from juvenile birds at 4 different ages: posthatch day (P) 10, P30, P50, and P70. I assayed males and females, n=3 for each age. Birds were collected from our animal facility and overdosed with isofluorane. Muscles were rapidly extracted and drop-fixed in 4% paraformaldehyde in 0.01 M PBS overnight at 4°C, then embedded in gelatin (8% in PBS). Gelatin cubes were refixed in 4% paraformaldehyde for 2 hours at room temperature, then cryoprotected in 30% sucrose in 0.01 M PBS at 4°C until sinking. Gelatin cubes were nicked with a razor blade for post hoc orientation and sidedness identification, mounted in OCT media (Fisher) and cryo-sectioned at 30 μm thickness into a series of 4. Tissue was stored in PBS at 4°C until further processing. During collection, sex was denoted by visual inspection of the gonads, and trunk blood and liver samples were also collected for genomic sexing for further confirmation as needed.

Fluorescence Immunohistochemistry.

The protocol was performed on all sections from a single series, at room temperature on a rotating mixer unless otherwise noted. Tissue was rinsed in 0.01 M phosphate buffered saline (PBS) for 30 minutes, then blocked in 5% bovine serum albumin (BSA) for 1 hour. One series was incubated for 24 hours at 4°C in primary antibody solution: PBS supplemented with 0.3% Triton X-100 and 2% BSA (PBT+), including mouse anti-neurofilament (NEFM; 1:500, Thermo No.13-

0700). Sections were rinsed extensively in PBT+, then incubated in the secondary antibody solution for 2 hours: α-bungarotoxin Alexa Fluor 555 (BTX; 1:100, Invitrogen No.A32728), goat anti-mouse Alexa Fluor 647 (1:500, Invitrogen No.A32728), and Wheat Germ Agglutinin Alexa Fluor 488 (WGA; 1:250, Thermo No.W11261). Sections were extensively rinsed in PBS, mounted onto Superfrost Plus slides (Fisher), and briefly dried before being coverslipped using ProLong GOLD Anti-Fade (Fisher No. P10144). A second series was processed following a similar protocol, but with mouse anti-neurofilament replaced by mouse anti-MY32 (1:100, Thermo No.M4276). All other steps, solutions, incubation times, and temperatures were unchanged.

Imaging and Quantification.

To assess the morphology of muscle fibers and NMJs, I captured images using microscopes at the University of Chicago Light Microscopy Core. Images were captured on a Marianas Spinning Disk Confocal microscope (3i) with a 20x plan-neofluar 0.5 numerical aperture (NA) objective and a 100x alpha plan-fluar 1.45 NA objective. All samples were imaged with identical collection parameters. Z-stacks were captured at a step-size of 1μm for all tissues, and syrinx samples were captured as stitched mosaics of the entirety of tissue sections for efficacious identification of muscle groups. Z-stacks were flattened using the two-dimensional maximum intensity projection algorithm in ImageJ (NIH) before analysis. Images were hand-annotated and quantified using ImageJ for measurement. Given that I found no sex differences in adults based on muscle fiber group or hemisyrinx, I did not further subset developmental data and analyses in this way. Fiber size was calculated using the line tool in ImageJ, by measuring the diameter of individual muscle fibers nearby the visualized NMJs. NMJ area, circularity, and related measurements were calculated using the free draw tool in ImageJ to individually outline the visualized NMJs by hand. NMJs were only quantified when it was readily apparent there was no

superposition of multiple NMJs through the maximum intensity projections, with NMJ class noted by experimenter. All output measures were first averaged across all measures within a single bird, and these within-bird means were further used for statistical analyses.

Statistics.

Statistical tests were run with the anova function (analysis of variance; ANOVA) with Type III analysis in R (R version 3.5.1, stats package v 3.6.0) with $\alpha = 0.05$. These analyses were for main effects of sex, age, NMJ class, and all two-and three-level interactions. Parametric post hoc analyses were performed using the TukeyHSD function in the stats package. The same statistical tests were run for the second series analyzing muscle fiber type.

Figures
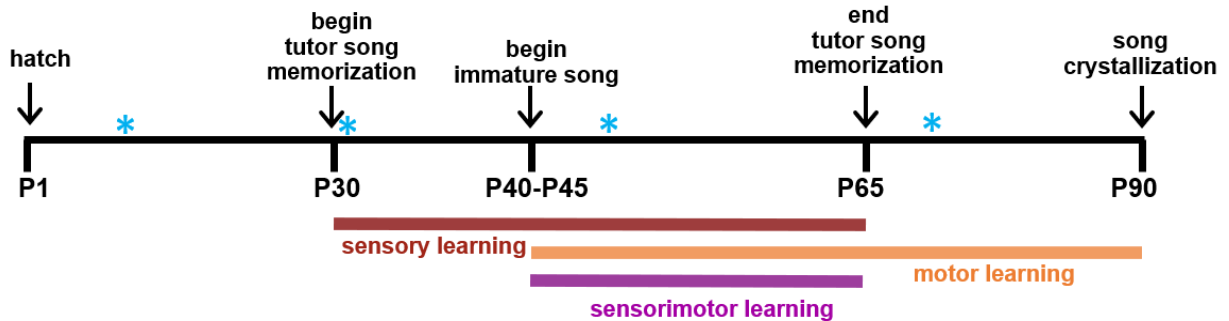
Figure 3-1: Timeline of Zebra Finch Vocal Development. Young zebra finch males begin to robustly memorize adult male tutor song models at P30, the opening of the sensory learning phase. By around P40 or P45, young males begin to produce immature song-like vocalizations, which is considered the opening of the motor learning phase. By P65, hearing additional songs no longer affects song output and is therefore considered the closing of the sensory learning phase. This overlap of sensory and motor learning phases between P40/45 and P65 is thus considered the sensorimotor learning phase. There is evidence that juvenile females engage in the same kind of sensory learning phase that males do, despite not learning to sing, though there is a lack of study on female non-song vocal development. Blue asterisks represent sample collection dates for this study (P10; P30; P50; P70).
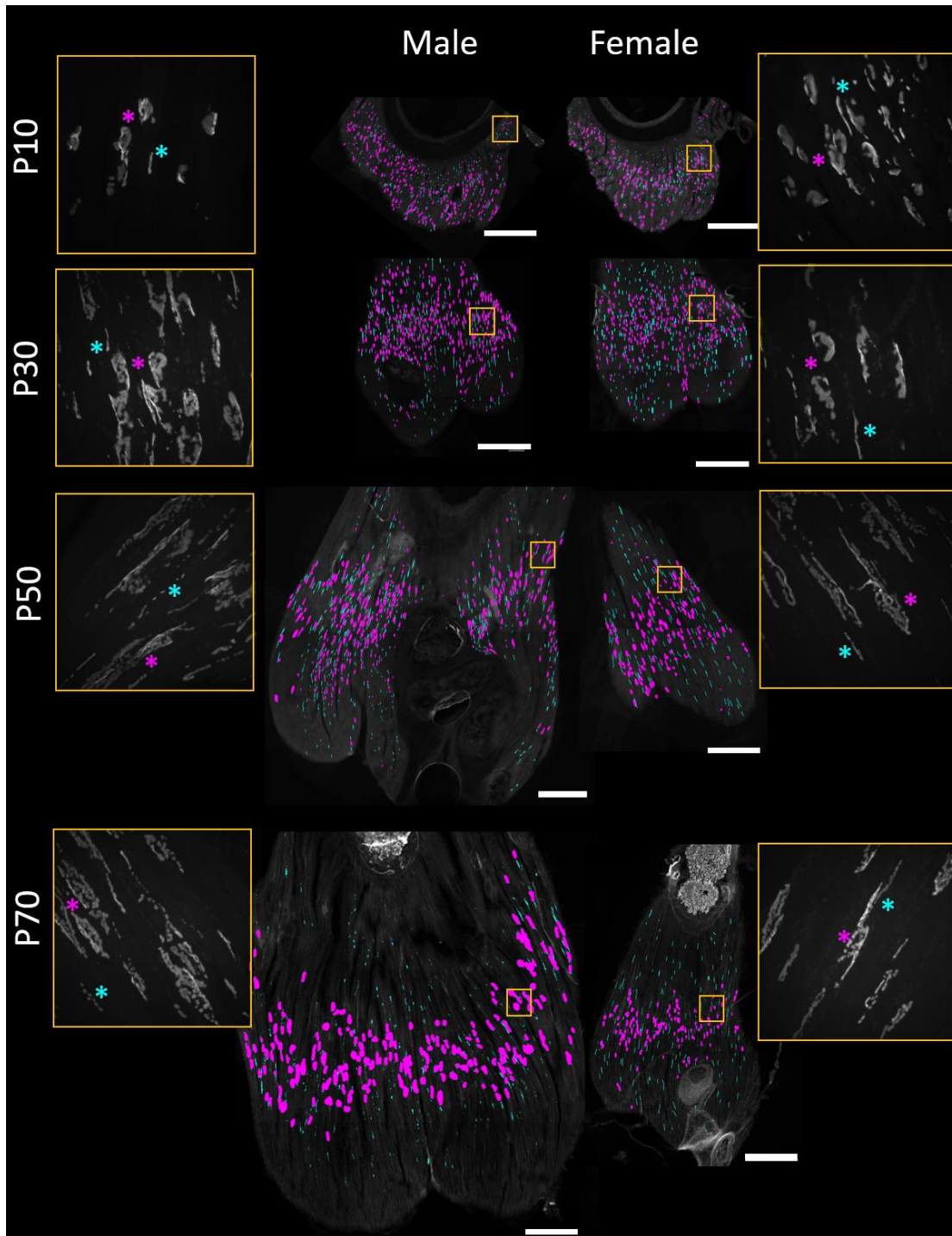
Figure 3-2: NMJs are not dimorphic early in life, and show sex-specific changes in morphology, size, and location across development. In early life, males (left) and females (right) are similar in the size, morphology, and distribution of *en plaque* (magenta) and *en grappe* (cyan) NMJs, as well as gross muscle size. Male muscles grow larger by P50 and more still by P70, while females do not. *en plaque* MEB organization starts by P50 and is adult-like by P70 in both sexes, while *en grappe* NMJs are predominantly lost outside the MEB in males, and grow slightly larger in these regions in females. Low-magnification mosaics were captured at 10x, and high-magnification insets (gold boxes) were captured at 40x. Scale bar is 200um for each.
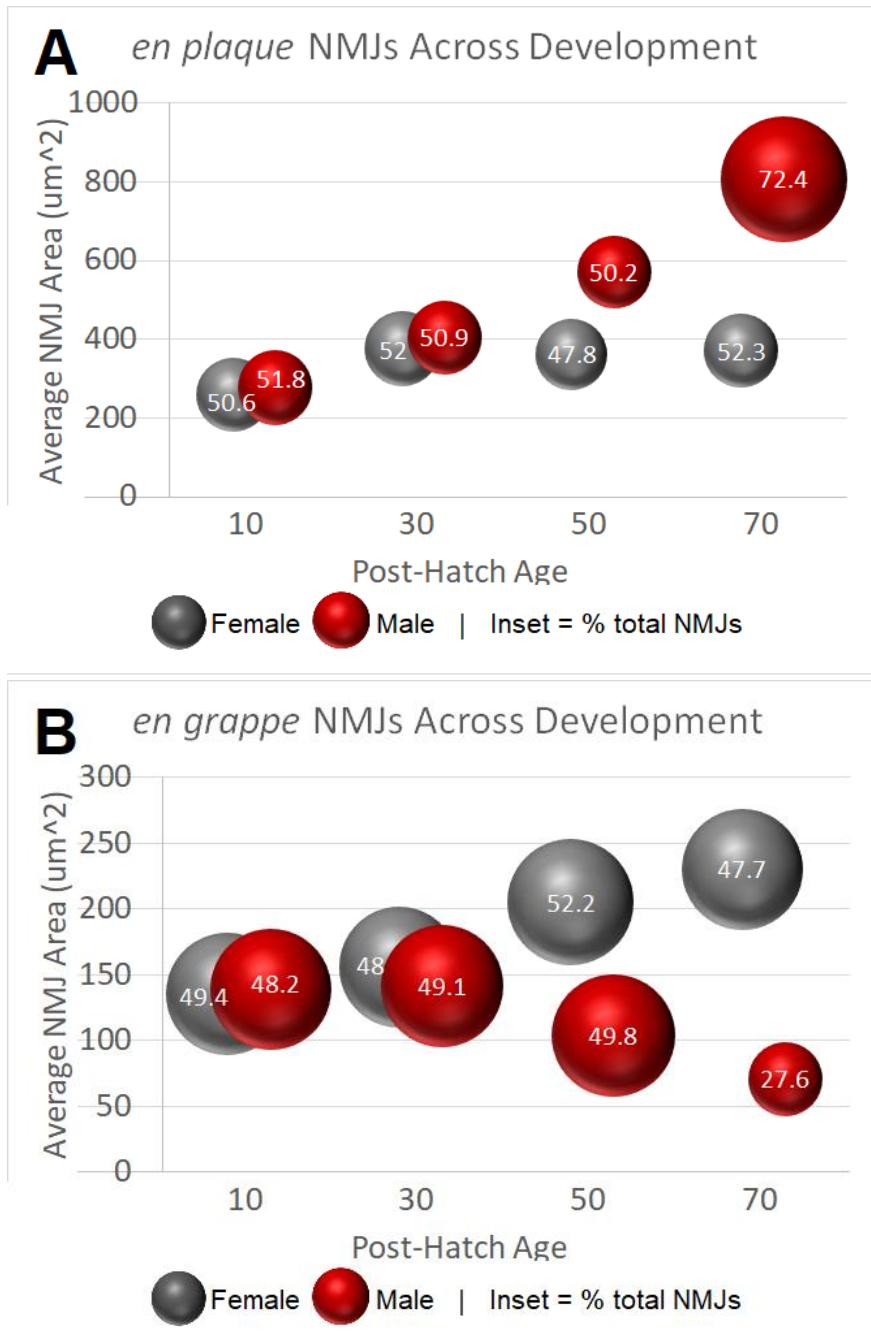
Figure 3-3: NMJs become sexually dimorphic in size by P50 and percentage by P70. A: Bubble plots of *en plaque* NMJs show sex-similarity early in development, both in size (y axis) and in proportion of total NMJs (bubble size; proportion inset to bubble size). Average *en plaque* NMJ size becomes larger in males by P50, though proportions are not sexually dimorphic. By P70, average *en plaque* NMJ size has grown even larger in males, and the proportion of NMJs has shifted to predominantly *en plaque*. Females are largely unchanged from P30 onwards. **B**: Bubble plots of *en grappe* NMJs show sex-similarity early in development. By P50, male *en grappe* NMJs have gotten smaller, while in females they have gotten larger. By P70, they have continued to grow larger and smaller in females and males, respectively. Axes, scales, and color codes are the same as in A.
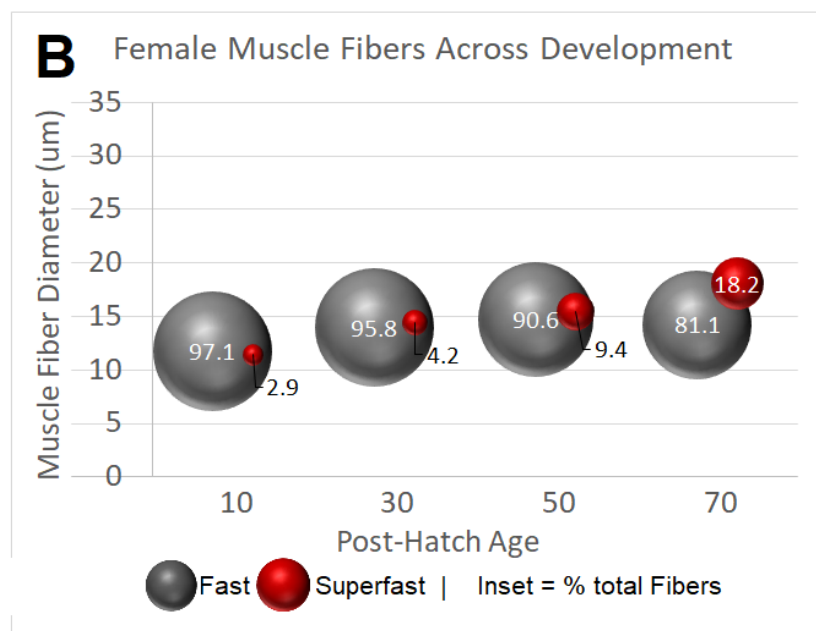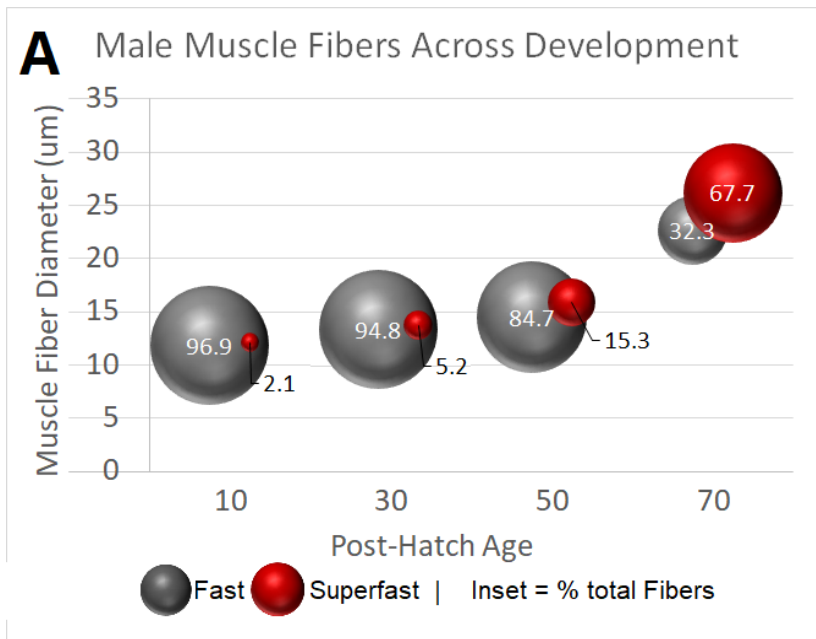
Figure 3-4: Muscle fiber types begin shifting in both sexes by P50 and reach their adult-like state by P70. A: Bubble plots of muscle fiber types across development in males. Early in life, the male syrinx is predominantly the weaker fast muscle fiber type (bubble size; proportions inset). By P50, superfast muscle fibers have increased, with moderate increases in muscle fiber size of both types. By P70, both fast and superfast muscle fibers have increased in size, and superfast has become the predominant muscle fiber type, nearing adult-like levels. **B**: Fast muscle fibers reach their adult-like size by P30 in females. Similar to males, by P50 there is a moderate increase in proportion of superfast muscle fibers. By P70, the size and proportion of superfast fibers has further increased and reached adult-like levels. Axes, scales, and color codes are the same as in A.

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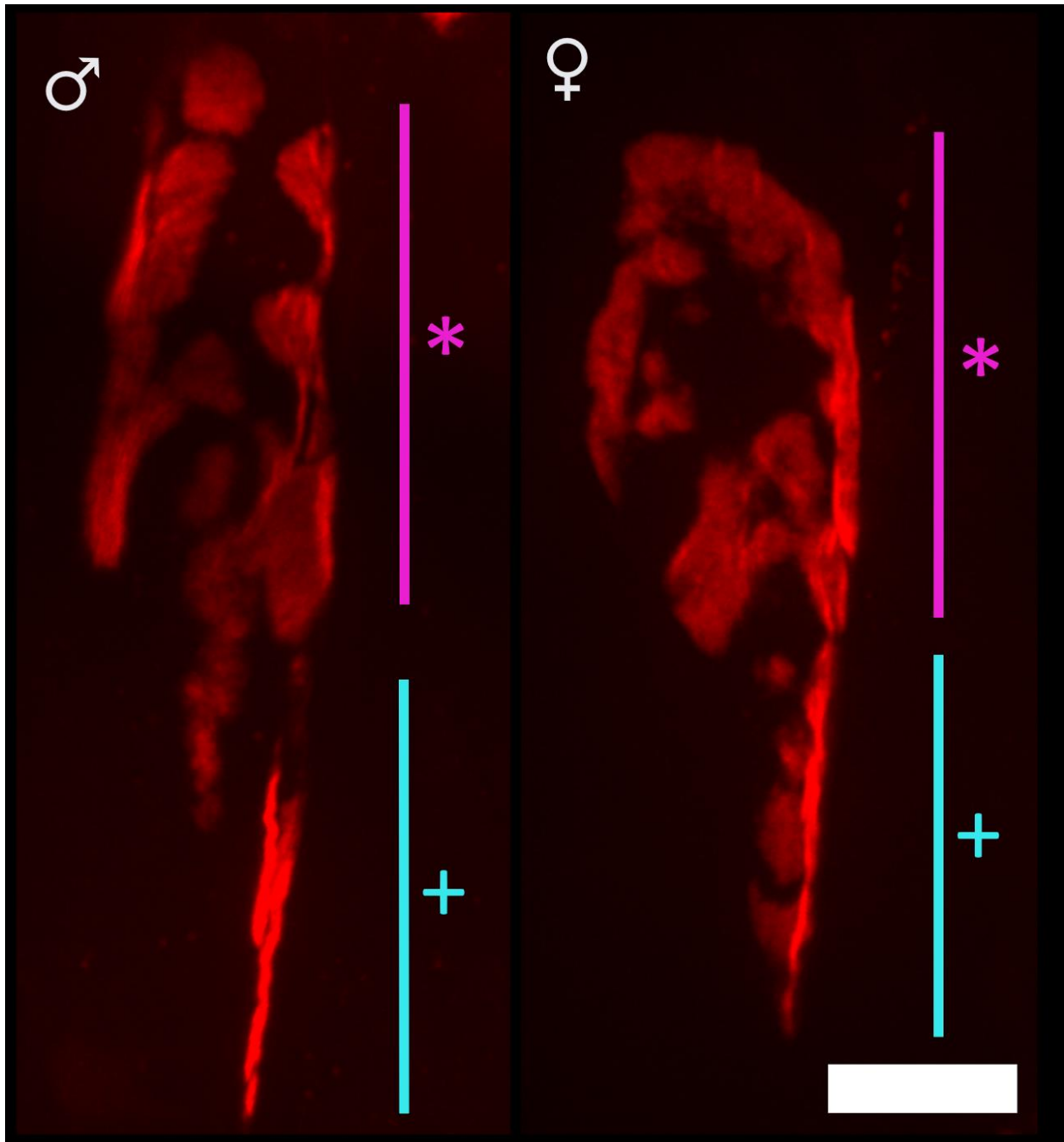
Figure 3-5: Intermediate NMJs are present during development in both sexes. Shown are maximum intensity images of male (left) and female (right) NMJs stained with a-bungarotoxin (synaptic receptors, red). These synapses have features of both *en plaque* (magenta; *) and *en grappe* (cyan; +) NMJs, indicating they are possibly in transition from one class to the other, though the direction of that transition is unclear. These representative images are from P30 samples. Images were captured with a 100x objective; scale bar is 20um.
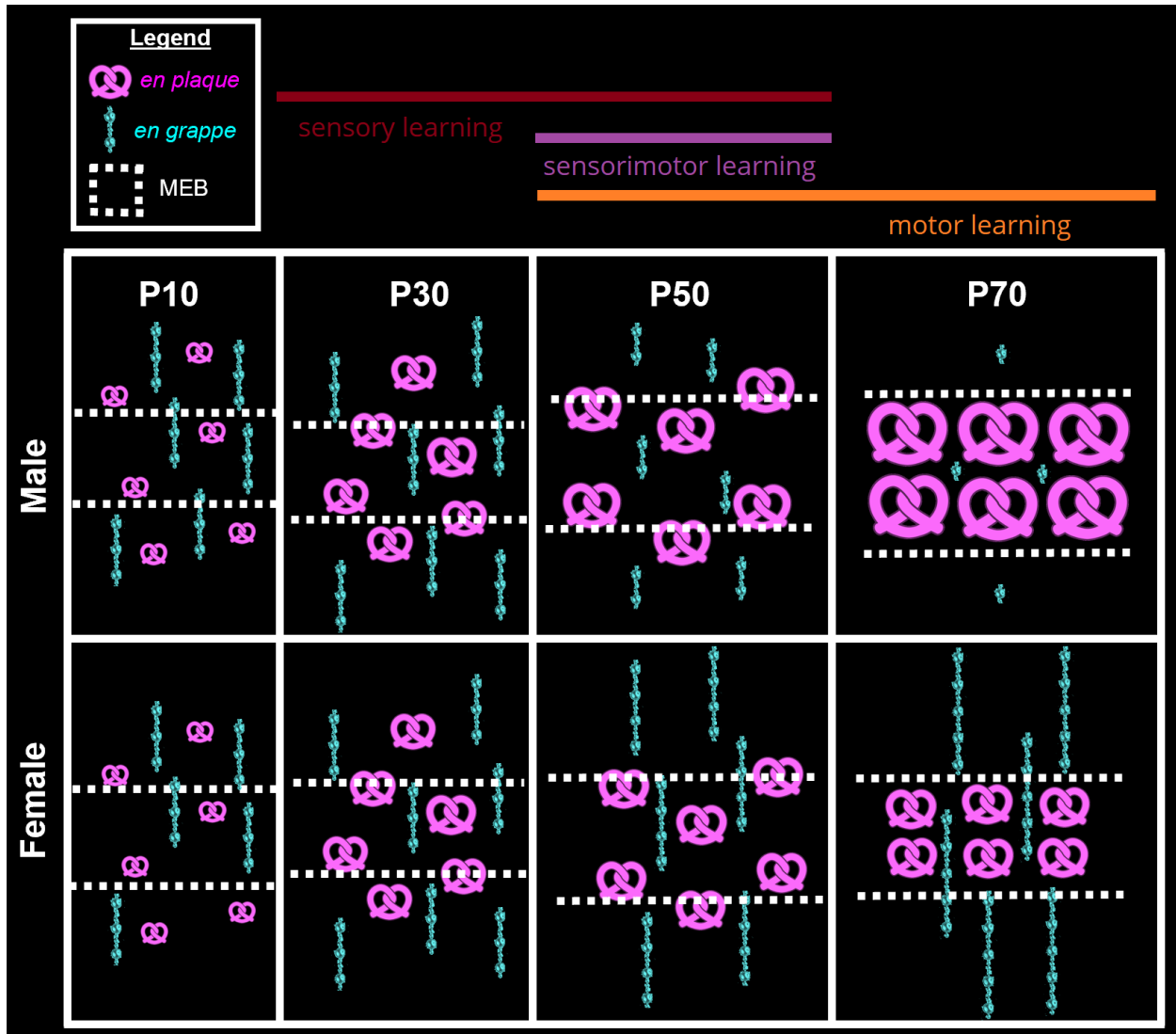
Figure 3-6: Cartoon summary of male and female NMJ development. Early in development, males and females are similar in proportions, relative size, and lack of organization of NMJ classes. By P30, *en plaque* NMJs have grown similarly larger in both sexes, and have begun to reorganize into a loose MEB, but proportion of classes has not changed. By P50, *en grappe* NMJs have gotten larger in females and smaller in males, while *en plaque* NMJs form a tighter but incomplete MEB in both sexes, only increasing in size in males. By P70, *en grappe* NMJs have gotten further larger in females and further smaller in males, while the *en plaque* MEB formation and size have neared the adult-like state in both sexes.

Chapter IV: Syrinx Synaptic Organization and Morphology Change with Hormonally Induced Changes in Vocal Behavior

Introduction

The hormonal milieu influences multiple features of vertebrate NMJs and their development, (muscle size, number and size of muscle fibers, muscle fiber types, and sizes of NMJs). These have been reported broadly in rodent locomotor muscles (Balice-Gordon et al. 1990b). The bulk of this work has focused on male-typical androgens, regarded as a positive regulator of muscle size and function. In contrast, there is relatively limited work on female-typical estrogens and their role in muscular physiology, though some evidence shows they reduce firing rates of innervating motor neurons (Wu et al. 2001), but rodent work shows variable estrogen levels in adulthood may not change muscular physiology (Lenell and Johnson 2021). Given the paucity of study on either estrogens or females the specific contributions of hormonal systems to variability in NMJ structure, muscle function, and behavioral output is yet unclear.

The developmental hormonal sensitivity of the zebra finch syrinx is a particularly useful framework to answer these questions. In this species, hormonal treatment early in development induces sex reversal of singing behavior. Juvenile females supplemented with estradiol (the main bioactive estrogen; "E2") throughout the period of song learning, produce male-like songs as adults but their syrinx does not grow to the size found in males (Wade et al. 2002; Simpson and Vicario 1991a; Gurney et al. 1982). In addition, E2 treatments in males throughout development does not hypermasculinize song circuitry or singing behavior, but does have a negative effect of

the syrinx in both weight and muscle fiber size (Wade et al. 2002). How syrinx NMJs change with hormone therapy, e.g., in masculinized females, in support of song production, is wholly unknown.

Similarly, juvenile males may be induced to become more female-like with developmental hormonal manipulations. Developmental treatment with exemestane, an inhibitor of aromatase (the enzyme that converts androgens into estrogens) has been shown to depress male song-learning and production ability (Choe et al. 2021), despite little to no effects on brain anatomy or gene expression. Interestingly, similar treatments make both male and female syringes more intermediate between a normal adult male and female (Wade et al. 2002). However, how NMJs change in feminized males, and how that might change to support repressed song learning and production, is also unknown.

Chapter 3 demonstrates that both males and females undergo active rearrangement of syringeal NMJs to reach their adult phenotype - not that females are simply undeveloped males. Given the behavioral changes developmental hormone treatments have on singing in this species described above, an obvious question is how hormonally mediated synaptic rearrangement in the syrinx might support these behavioral changes. One possibility is that the prevalence of *en grappe* NMJs in normal females is directly tied to suppressed singing ability, and either feminization of males or masculinization of females shifts the relative proportion of these synapses higher or lower, respectively. Another possibility is that song learning and production does not depend critically on synaptic composition or size at syrinxes, across sexes, and is instead wholly instantiated in brain circuits. This is a distinct possibility, given that early E2 treatment of females masculinizes the brain and singing behavior, but does not seem to masculinize gross features of the syrinx (Simpson and Vicario 1991a). A first step in determining between these possibilities is

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to see whether hormonal manipulation that swaps sex specific behavior also changes the synaptic composition at syrinxes, i.e., do NMJs change in each sex correlated with the induced behaviors?

Results

Summary

The data presented here are preliminary and qualitative, and the production and collection of additional experimental birds are ongoing. I have currently collected n=3 birds that have been treated with exemestane throughout development (1 male and 2 females), and n=4 birds that have been treated with estradiol (2 males and 2 females). Multi-color labeling, imaging, and analysis was done as described in preceding chapters, at post-hatch day (P) 120, when songs were crystalized and age-matched for birds that did not sing (Figure 1).

I found 4 main results, summarized here and further explained in depth in following sections:

1. Estradiol treatment masculinizes female syrinx muscle fiber size and composition, but moderately feminizes these features in males, despite both sexes producing song

2. Exemestane treatment makes male and female syringes intermediate to their normal adult counterparts in terms of muscle fiber size and composition

3. The size and MEB organization of *en plaque* NMJs is positively related to song

4. The presence of *en grappe* NMJs *only* outside the MEB region is positively related to song suppression

Hormone Manipulation Effects on Song Behavior, Gross Syrinx Morphology, and Muscle Fibers.

E2 treatments throughout development have been shown by many others to masculinize females, allowing for developmental song learning and adult stereotyped song production. I show similar effects in my hands, that females become masculinized both in plumage (Figure 2) and in singing behavior (Figure 3). Similar treatments in males have been shown by others not to "hypermasculinize" song behavior, and indeed I find this to be the case in terms of vocal behavior. I refute anecdotal reports from others that E2 does not seem to change the size of the female syrinx, but I find that males given E2 have a moderately more female-like syrinx in terms of gross size (Figure 4). This is supported by a lack of sexual dimorphism in muscle fiber size (Figure 6), though it is worth noting the average muscle fiber size in E2-treated males here is slightly smaller than in normal untreated adult males, and in E2-treated females, slightly larger than normal untreated adult females. Interestingly, these treatments do lead to a sexual dimorphism in terms of muscle fiber type. While both sexes sing a species-typical song, females treated with E2 have a predominance of superfast muscle fibers, while males treated with E2 have a higher proportion of fast muscle fibers that is less than a normal male (Figure 6).

Exemestane treatments throughout development have been shown by others to feminize males to an extent, depressing song learning and production, as well as some loss of male-typical plumage development (Choe et al. 2021). I show similar results in my hands (Figures 2, 3). I also confirm reports from others that aromatase inhibitors make both male and female syringes more intermediate between normal adult males and females in terms of gross size (Figure 4). However, this similarity in gross size is counter to average muscle fiber size, with females having moderately smaller average muscle fibers than males (Figure 6), suggesting that developmental addition of muscle fibers in the syrinx might be sensitive to hormonal milieu. Interestingly, these treatments also shift proportions of muscle fiber types relative to normal adults, but the result is not sexually

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dimorphic. Both males and females treated with exemestane have approximately equal prevalence of fast and superfast fibers (Figure 6).

en plaque NMJ Size and MEB Organization Positively Scale with Song Behavior.

When given developmental E2 treatments, both males and females learn to sing. I find that the average *en plaque* NMJ size is not sexually dimorphic after these treatments (Figure 4), and is near or above normal adult male size. The *en plaque* NMJs in both sexes also form a typical MEB. In contrast, developmental exemestane treatments depress male song learning and production, and may lead to some amount of song-like vocalizations in females (Choe et al. 2021). Here, I find again a lack of sexual dimorphism in average *en plaque* NMJ size (Figure 4), though these measures are intermediate to normal adult males and females. The *en plaque* NMJs in both sexes also form a weaker MEB, with many *en plaque* NMJs found outside the canonical MEB region in both sexes.

en grappe NMJs Outside the MEB Positively Scale with Song Suppression.

In normal adults, males sing and females do not and females have many *en grappe* NMJs outside the MEB region and males do not. When given developmental E2 treatments, both sexes sing and I find that there are a number of *en grappe* NMJs within the MEB in both males and females, and not sexually dimorphic in average size or proportion (Figure 5). I also find that outside the MEB region, there are also *en grappe* NMJs in both males and females (Figure 4). However, these *en grappe* NMJs tend to be small and fractured, and to a first approximation appear visually similar to the extra-MEB *en grappe* NMJs found in P70 males (see Chapter 3). In contrast, when given developmental exemestane treatments, I find that there are numerous *en grappe* NMJs, particularly in the region outside the canonical MEB, in both sexes (Figure 4). These *en grappe*

NMJs, to a first approximation, appear visually similar to the typical adult female *en grappe* NMJs in this region.

Discussion

In the zebra finch, hormonal manipulations induce sex-reversal of song behavior, either spurring females to sing or reducing male vocal ability. Whether these brain and behavioral changes broadly extend to the synaptic components of the vocal organ was unknown. I show here preliminary indications from hormonal manipulations that the presence of *en grappe* NMJs outside of the canonical MEB region of the syrinx might be directly tied to song production ability. In contrast, muscle size and fiber type composition are not as tightly correlated to song ability. The implication of these results is that there is a complex interplay between hormonal milieu, neuromuscular anatomy, and behavioral output - and indicate that the interaction of NMJ class and location might be the anatomical substrate by which singing ability is either promoted or suppressed.

When song is promoted, either through normal male development or E2 treatments in development for both males and females, the most obvious synaptic outcomes are in the size of *en plaque* NMJs and their organization into a MEB. These data are highly similar to what I reported in Chapter 2 for adult males, and would imply the size and organization of *en plaque* NMJs specifically is correlated to song production. NMJ class proportion is near normal adult male proportions, but there are still a number of *en grappe* NMJs in either treatment group. These *en grappe* NMJs found outside the MEB are small in both number and size, and to some first approximation appear visually similar to the fragmented extra-MEB *en grappe* NMJs found in P70 males. Interestingly, I also find *en grappe* NMJs within the MEB for both treatment groups, which are less fractured than those outside the MEB, and appear visually similar to the *en grappe* NMJs

found in normal adult females. This would imply that not solely the growth of *en plaque* NMJs, but the growth of any NMJ and their centralization into a MEB might be the substrate of song production ability. More rigorous analyses across multiple birds, considering sizes and proportions of these classes both within and outside the MEB region, would be necessary to disentangle the relative contributions of these two features to the behavior, and are planned.

The behavioral results observed with exemestane treatment (repressed male song, some song-like vocalizations in females) correlate with NMJ sizes and proportions. Both males and females have similar sizes and proportions of *en plaque* NMJs, intermediate to normal males and females, though closer to normal males. These *en plaque* NMJs form a crude MEB with numerous *en plaque* NMJs outside the canonical MEB region. Interestingly, this treatment in males also leads to many *en grappe* NMJs being found outside the MEB which are larger than those reported for E2 treatments. While more rigorous analyses are necessary, the implication here is that the number and size of *en grappe* NMJs, specifically outside the MEB region, are positively correlated to song suppression - as *en grappe* NMJs outside the MEB are lost, more typical song ability emerges; as they emerge, more typical song ability is lost.

Interestingly, muscle fiber type does not appear as related to singing ability as NMJ class and location. Normal and E2-treated males both sing typical songs, yet superfast fibers are predominant only in the normal males, being evenly split with fast fibers in E2-treated males. This is in contrast to E2-treated females, who also sing male-typical songs, but who have a predominance of superfast muscle fibers similar to normal males. Song is only one vocalization a normal adult male produces, in addition to many other types of call behaviors. Analysis of muscle fiber type across songbird species by others has indicated that superfast muscle fiber proportion might not be directly related to singing ability, but instead to the full variety of the vocal repertoire

(Christensen et al. 2017). In normal adult male zebra finches, song is the primary vocalization, accounting for more than half the daily vocalizations. Whether this is true of E2-treated males and females is unknown, though could account for the variability I see in superfast muscle fiber percentage despite little variability in ability to sing. Circulating hormones are also related to song production, insofar as variable circulating hormone levels are correlated with the amount of total song being sung across a time period (Adkins-Reagan 1999, Williams et al. 2003, Wang et al. 2014). Indeed, in prior reports of E2-treatments in female zebra finches, some of these birds need acute testosterone treatment in adulthood to spur them to produce the learned songs (Simpson and Vicario 1991). It is possible that circulating hormone levels, in conjunction with total proportion of vocalizations being song vs. calls, could account for the variability in muscle fiber type reported here. More extensive analyses of all vocal behavior in these E2-treated birds, as well as measurement of circulating hormone levels, would be necessary to make a stronger claim and are planned.

The presence of some amount of *en grappe* NMJs outside the MEB, though small, in E2-treated males and females is curious. In Chapter 3, I showed that these features are found in P70 males, making an argument that further refinement of song to its adult crystallized state might be the mechanism by which these fragmented *en grappe* NMJs outside the MEB are lost by normal male adulthood. This might indicate that in our E2-treated birds, song is not yet as crystallized as initially thought. It is well-known that manipulations of developmental song learning do shift the closure of these learning phases. For instance, both isolation from hearing song and early-life deafening, lead male song crystallization after the typical transition to adulthood, extending this closure up to months (Mori and Wada 2015). Further analyses of song behavior would be useful to determine what kind of variability in song behavior might be present in our birds, and whether

that speaks to the presence of these small *en grappe* NMJs outside the MEB. It is also possible that these *en grappe* NMJs outside the MEB are instead a consequence of high circulating E2 levels in our treatment groups. The exact effects of sex hormones on NMJ class prevalence are poorly understood, both in our model and across other vertebrates.

The relative contribution of song ability, total amount of singing, and circulating hormone levels to these extra-MEB *en grappe* NMJs could be addressed in two ways. First, the E2 implants are continuously releasing small amounts of the hormone across the lifespan, but could be removed once birds have reached sexual maturity. Then, after a short period of time to allow circulating hormone levels to return to normal, these birds could be collected and assayed. A lack of *en grappe* NMJs outside the MEB here would indicate that the presence of this NMJ class in this region is more directly tied to circulating E2 and its downstream effects. This could potentially be paired with acute E2 treatments in normal adult males and estrogen receptor antagonists in normal adult females for further verification. Second, future collections could be done at later timepoints into adulthood, much longer after song has been crystallized. A lack of *en grappe* NMJs outside the MEB later into adulthood would indicate some nuance in the relationship of NMJ features and song crystallization that could then be further uncovered.

Methods

All procedures were conducted in accordance with the National Institute of Health guidelines for the care and use of animals for experimentation and were approved by the University of Chicago Institutional Animal Care and Use Committee.

Experimental Animals and Hormonal Treatments.

All birds used in this study were bred at the University of Chicago, housed on a 14h:10h light:dark cycle, with seed and water provided *ad libitum*. Breeding animals were kept in a group-housed environment, with no more than 3 adult pairs per large cage, with a total of 6 single-cage boxes utilized. Each box was used for a single hormonal treatment regimen, to prevent cross-contamination. Juveniles were tagged with leg bands for individual identification when fledged, and in cases where many juveniles fledged at once and pushed the cage to capacity, some juveniles were moved either to a small cage in the same box, or into a separate box to be fostered by other adult pairs. Neither has been shown to robustly affect developmental song learning in this species. After any movement was done (always by P30), experimental birds were kept in the same environment until adulthood and songs were crystallized.

Pharmacological preparations and treatment timelines.

Exemestane (Sigma PHR1634) is an inhibitor of aromatase, the sole enzyme responsible for converting androgens into estrogens. Exemestane was first dissolved in DMSO (Invitrogen D12345) to a concentration of 100mg/mL, then this solution was suspended in olive oil (Sigma 75343) to a final concentration of 2mg/mL. Suspending in oil is used to prolong absorption of the compound, and has been validated in other reports, as has the efficacy of exemestane in the zebra finch model. Exemestane treatments were given starting at hatch by applying one drop (~50uL) of the working solution topically, near the flank or under the wing. These treatments were given daily until P65, then every other day until sacrifice.

Estradiol ("E2", Sigma E1024-1G) is the main bioactive form of estrogen, and is well utilized in the zebra finch model. E2 preparations for topical treatments were made in the same way as described for exemestane, first dissolved in DMSO to a concentration of 100mg/mL, and further suspended in olive oil to 1mg/mL. Additionally, subcutaneous implants were made by

mixing the 100mg/mL E2 stock solution with medical grade silicone adhesive. The mixture was extruded from a needle into silastic tubing and allowed to cure overnight, after which implants were cut and weighed so that each implant contained approximately 200ug of estradiol, and kept in sterile conditions until use. Topical treatments for E2 were given in a similar manner as exemestane, daily until P14, then alternating daily until P20. At P20, topical treatments stopped and juveniles were given E2 implants, subcutaneously either under the wing or in the middle of the back near the shoulder blades.

Prior to silastic implant, juveniles were given meloxicam intramuscularly at 0.3mg/kg body weight, then anesthetized with 3.5% isofluorane in 100% oxygen, which was sustained at 1.5% during the surgery. Surgical sites were plucked and scrubbed with 10% providone-iodine. An incision was made in the skin, and a blunt hemostat used to create a pocket under the skin where the implant was placed. Incisions were closed with veterinary adhesive (VetBond, 3M) and 4% lidocaine applied topically. Juveniles were observed for 1.5 hours after surgery to ensure recovery, and were returned to their home cages after they began flying and attempting to eat. They were observed daily afterwards to ensure no issues in recovery, and to ensure the implants were maintained until adulthood collection.

Sample collection.

All animals were collected between 11a and 1p, or approximately 4-6 hours after lights on. Animals were overdosed with isofluorane followed by rapid decapitation. Tissues were quickly extracted and drop-fixed in 4% PFA in PBS at 4°C. Trunk blood was collected at sacrifice, first left to clot at room temperature for 20-30 minutes, then centrifuged at 16,4000g for 10 minutes to separate serum from cells. The serum was stored at -80°C. Sex was confirmed by post-mortem

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examination of the gonads, and liver samples were collected and stored at -20°C for further confirmation of genomic sex.

Drop-fixed samples were stored in 4% PFA in PBS at 4°C overnight, then embedded in gelatin (8% in PBS). Gelatin cubes were refixed in 4% paraformaldehyde for 2 hours at room temperature, then cryoprotected in 30% sucrose in PBS at 4°C until sinking. Gelatin cubes were mounted in OCT media (Fisher) and cryo-sectioned at 40 μm thickness into a series of 4. Tissue was stored in PBS at 4°C until further processing.

Fluorescence Immunohistochemistry.

The protocol was performed on all sections from a single series, at room temperature on a rotating mixer unless otherwise noted. Tissue was rinsed in 0.01 M phosphate buffered saline (PBS) for 30 minutes, then blocked in 5% bovine serum albumin (BSA) for 1 hour. One series was incubated for 24 hours at 4°C in primary antibody solution: PBS supplemented with 0.3% Triton X-100 and 2% BSA (PBT+), including mouse anti-neurofilament (NEFM; 1:500, Thermo No.13-0700). Sections were rinsed extensively in PBT+, then incubated in the secondary antibody solution for 2 hours: α-bungarotoxin Alexa Fluor 555 (BTX; 1:100, Invitrogen No.A32728), goat anti-mouse Alexa Fluor 647 (1:500, Invitrogen No.A32728), and Wheat Germ Agglutinin Alexa Fluor 488 (WGA; 1:250, Thermo No.W11261). Sections were extensively rinsed in PBS, mounted onto Superfrost Plus slides (Fisher), and briefly dried before being coverslipped using ProLong GOLD Anti-Fade (Fisher No. P10144). A second series was processed following a similar protocol, but with mouse anti-neurofilament replaced by mouse anti-MY32 (1:100, Thermo No.M4276). All other steps, solutions, incubation times, and temperatures were unchanged.

Imaging and Quantification.

I captured images using microscopes at the University of Chicago Light Microscopy Core, on a Marianas Spinning Disk Confocal microscope (3i) with a 20x plan-neofluar 0.5 numerical aperture (NA) objective and a 100x alpha plan-fluar 1.45 NA objective. All samples were imaged with identical collection parameters. Z-stacks were captured at a step-size of 1µm and as stitched mosaics of the entirety of tissue sections for efficacious identification of muscle groups. Z-stacks were flattened using the two-dimensional maximum intensity projection algorithm in ImageJ (NIH) before analysis. Images were hand-annotated and quantified using ImageJ for measurement. Fiber size was calculated using the line tool in ImageJ, by measuring the diameter of individual muscle fibers nearby the visualized NMJs. NMJ area, circularity, and related measurements were calculated using the free draw tool in ImageJ to individually outline the visualized NMJs by hand. NMJs were only quantified when it was readily apparent there was no superposition of multiple NMJs through the maximum intensity projections, with NMJ class noted by experimenter. All output measures were first averaged across all measures within a single bird.

Figures
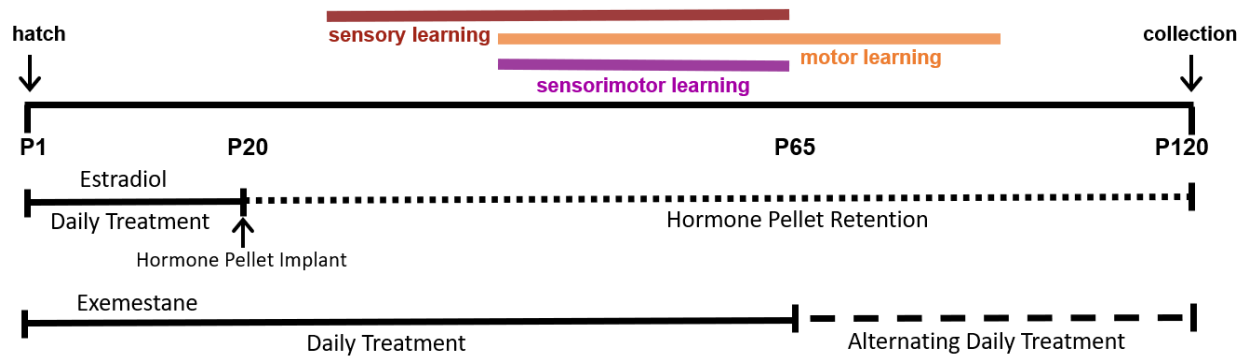
Figure 4-1: Experimental Hormonal Manipulation Timeline. All birds began receiving treatments within 24 hours of hatch. For the first 20 days of life, chicks received daily topical application of either estradiol or exemestane. For birds being treated with estradiol, on P20 daily topical treatments were ceased and instead a pellet loaded with estradiol was implanted subcutaneously, to continuously release the hormone into the body. For birds being treated with exemestane, daily treatments were continued until P65, after which they were treated every other day until collection.

Figure 4-2: Hormonal manipulation effects on plumage are dependent on sex and treatment. Normal adult males and females differ in plumage, with males having orange cheek patches, black breast bars, and brown flanks speckled with white spots, while females lack these features. When treated with estradiol, male plumage does not change, but female plumage becomes male-like. When treated with exemestane, male plumage is lost but not completely absent, while female plumage does not change.
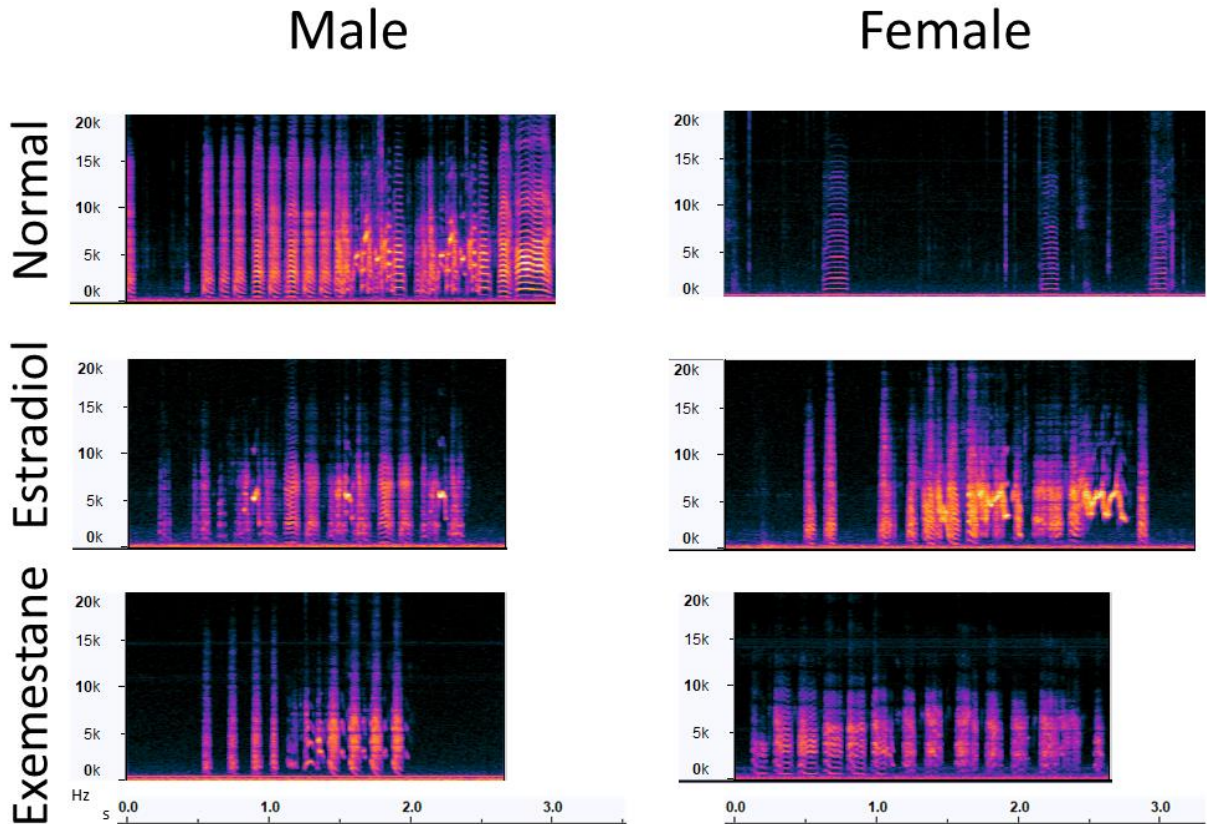
Figure 4-3: Hormonal manipulation effects on vocalizations. In normal adults, males sing complex songs while females only vocalize calls. When treated with estradiol, females learn and produce male-like song, while male song is largely unaffected. When treated with exemestane, male song is diminished, and spurs some song-like vocalizations in females.
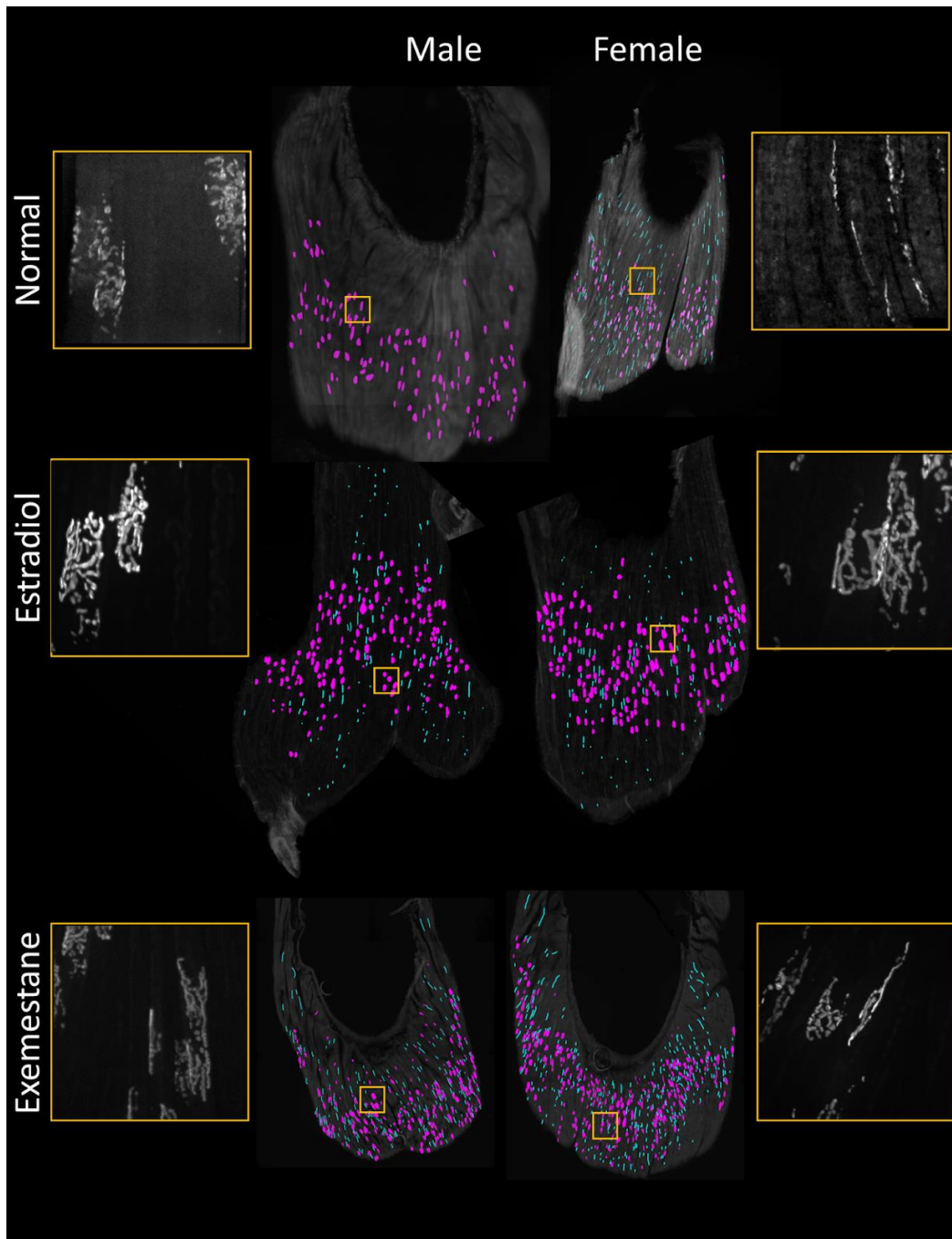
Figure 4-4: Hormonal treatment effects on syrinx size and NMJ organization. In normal adults, male syringes are 1-2x larger than females, and while an *en plaque* MEB is present in both sexes, it is the females alone that have a large number of *en grappe* NMJs more spatially disorganized. When treated with E2 throughout development, both males and females have syringes highly similar in size, with some small *en grappe* NMJs outside the canonical MEB region. When treated with exemestane throughout development, the syrinx becomes more intermediate to normal adults in both males and females. A loose *en plaque* MEB is formed, but many *en plaque* NMJs may be found outside this region. In addition, many more *en grappe* NMJs are present in both sexes. Low-magnification mosaics captured at 20x; high-magnification insets captured at 63x, denoted by gold box. Magenta *en plaque*, cyan *en grappe*.
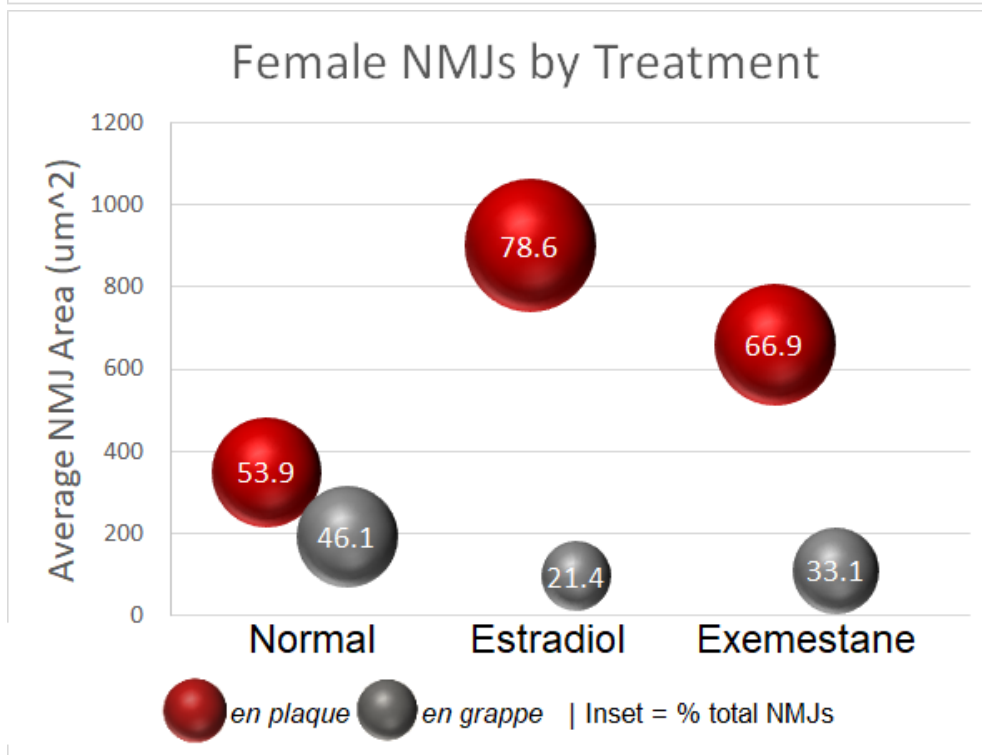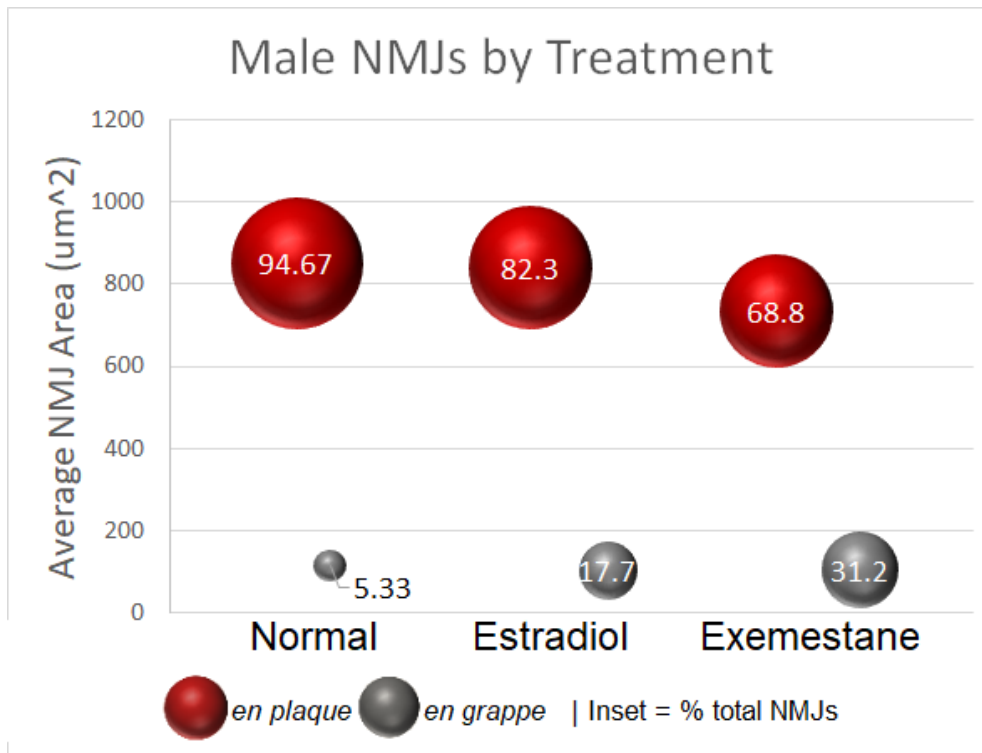
Figure 4-5: Hormone manipulation effects on NMJs indicate a relationship of *en grappe* NMJs to song suppression. E2 males still sing, and while there is a slight decrease in proportion of *en plaque* NMJs, average size does not change. Exemestane males show some song repression, and there is a decrease in both proportion and size of *en plaque* NMJs. The relative outcomes are recapitulated in females, both in terms of size and proportions of NMJs, as well as singing.
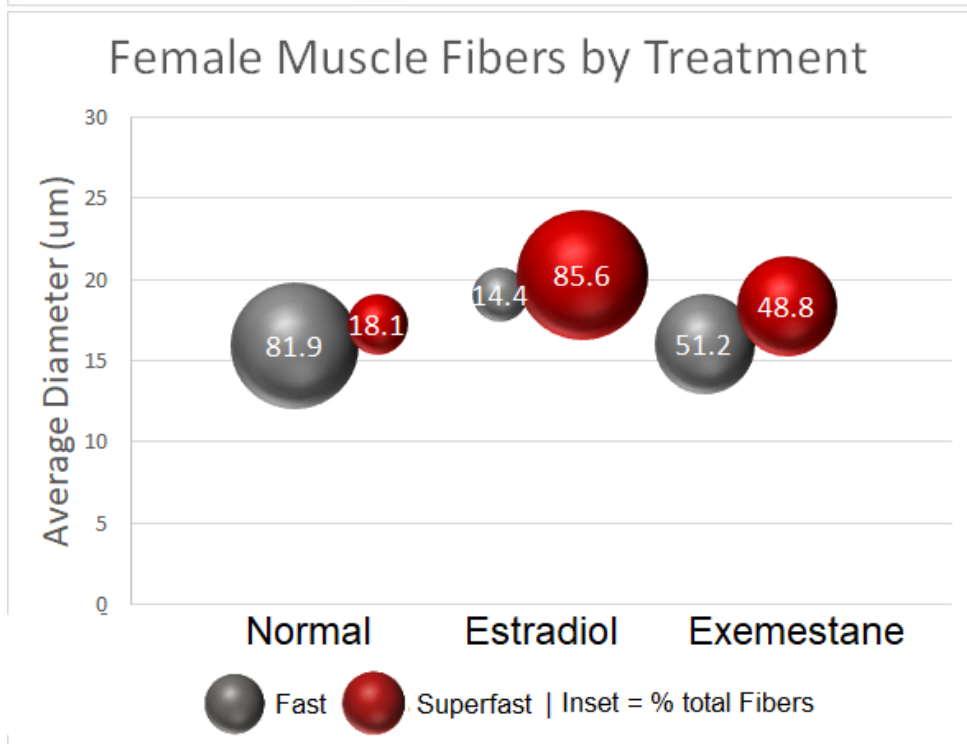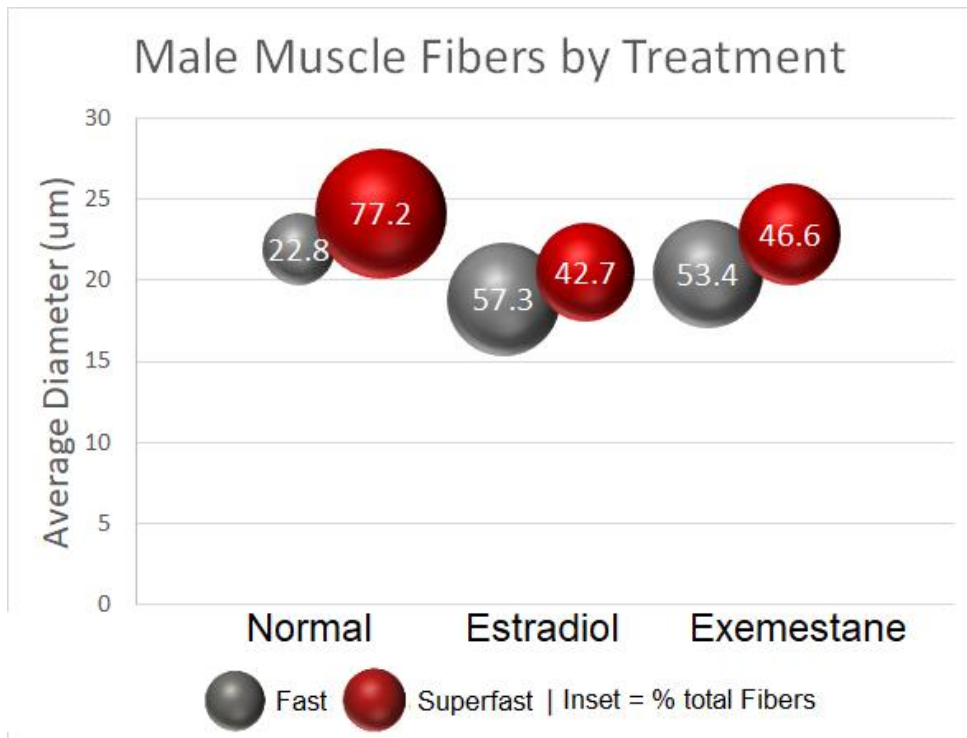
Figure 4-6: Hormone manipulation effects on muscle fiber types are less clear in their correlation to singing ability. Despite differences in singing ability, males treated with E2 or exemestane have similar sizes and proportions of superfast muscle fibers, both lesser than normal males. In contrast, the size and proportion of superfast muscle fibers is more correlated to singing ability in treated females.
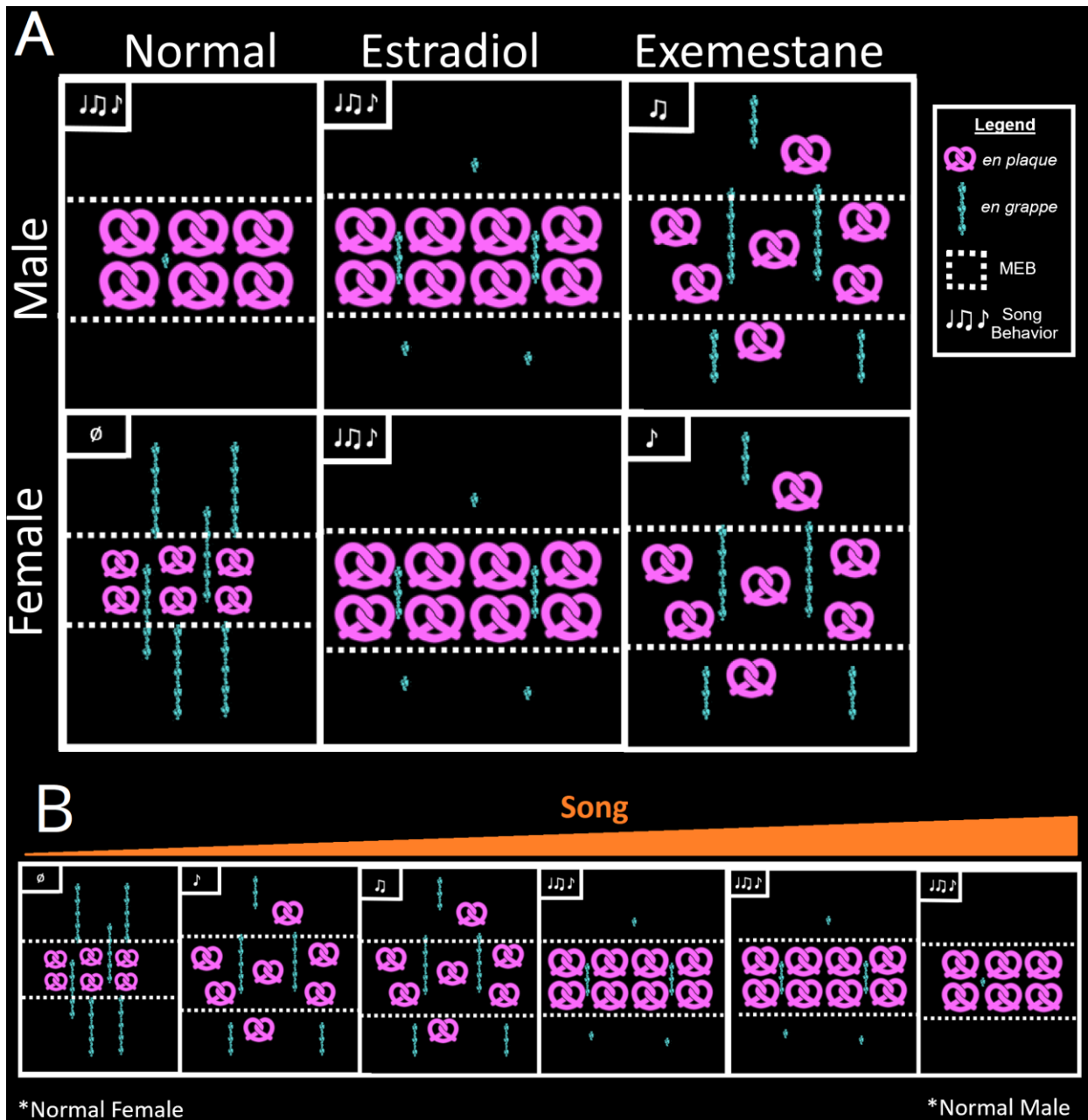
Figure 4-7: Cartoon summary of male and female NMJ outcomes in adulthood after developmental hormone manipulations. A: Normal males sing and have predominantly large *en plaque* NMJs organized into a MEB. Normal females also have this organization, though with smaller *en plaque* NMJs, and an equal occurrence of *en grappe* NMJs. When treated with exemestane, male song is repressed, *en plaque* NMJs are smaller and more loosely organized, and there are more *en grappe* NMJs. When treated with E2, male *en plaque* size and organization is no different from normal, though there are some vestiges of *en grappe* NMJs. These features are recapitulated in females within each treatment. B: Thus, *en plaque* size and organization seems to scale with song production ability in some but not all cases, but in particular, *en grappe* presence outside the MEB is more related to song suppression. As *en grappe* NMJs outside the MEB are lost, more typical song ability emerges; as these NMJs emerge, more typical song ability is lost.

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Chapter V: Discussion

Summary of Results and Implications

I have shown, first, that the synaptic organization of the zebra finch syrinx is uniquely sexually dimorphic, in a female-biased manner. In all non-vocal muscles measured, both males and females have near complete presence of the classic vertebrate *en plaque* NMJ. This includes the intrinsic tongue muscles, which receive innervation from the same motor pool that innervates the syrinx. In the syrinx, however, there is a rather extreme sexual dimorphism: males appear as every other muscle measured, nearly completely large *en plaque* NMJs, while females are equitable between *en plaque* and the smaller *en grappe* NMJs. These *en grappe* NMJs are biased to appear on smaller and weaker muscle fibers, suggesting their output is weaker than *en plaque*s. These *en grappe* NMJs additionally fail to form a motor endplate band, and instead are found throughout the muscle fiber length, implying the motor signals to initiate vocalizations are potentially more dispersed than in males. Finally, these *en grappe* NMJs are singly innervated, arguing that these synapses are actively developed, and not simply a juvenile or underdeveloped phenotype. Ultimately, these results argue that there is an active developmental program leading to this adult female phenotype, and one that is distinct from the male. They also argue that these features may be an anatomical substrate of female song suppression in this species. Female song is ancestral in songbirds, and highly common across exigent songbird species, though more recently lost in the zebra finch and some others (Odom et al. 2014), and these synaptic sexual dimorphisms in the muscles controlling vocalizations are likely one potential anatomical mechanism of an evolutionary loss of behavior.

I then demonstrated that these adult dimorphisms are ultimately realized over two divergent development programs, one male-specific and the other female-specific. Early in life, males and females are indistinguishable at every level measured, yet particularly different from either adult phenotype, indicating either sex might have the *potential* to develop towards an adult male-like or female-like syringeal synaptic morphology. This common starting place supports other developmental work on hormonal manipulations modifying syringeal structure and function, as our earliest age assayed (P10) is within the window by which hormonal manipulations must be given to females in order to make them grow up to produce male-like song (Simpson and Vicario 1991a). Curiously, I also find little differences at P30, an age at which the same hormonal manipulations fail to turn females into singing males (Simpson and Vicario 1991a), which could indicate there are sex-specific changes in the syrinx by this age that I have failed to uncover in these analyses. Through the remainder of development, synaptic rearrangements are sexually dimorphic, primarily in the male loss and the female enlargement of *en grappe* NMJs. These results further support the hypothesis that *en grappe* NMJ as the substrate of female song suppression. Given that males may be biased towards losing *en grappe* NMJs first in the region outside the MEB, this region and the presence of *en grappe* NMJs there may reflect a particular hinge in both the ability to sing and the ability to produce stereotyped song as adults.

Finally, I showed preliminary indications that song production might be tied to the interaction between NMJ class and location in the syrinx. Chronic developmental treatments with E2 allows females to learn and produce male-like songs, and their syrinx NMJs become highly similar to males in size and organization, including the loss of *en grappe* NMJs outside the MEB region. In contrast, similar treatments with exemestane, which prevents the conversion of androgens into estrogens, repress male singing ability, and make the male syrinx more female-like

- including an increase of *en grappe* NMJs outside the MEB. Thus, it is not solely the presence of *en grappe* NMJs, but their location outside of the canonical MEB, that is most correlated to song suppression.

Taken together, the results discussed here make a case for the female zebra finch being the specialized sex for song suppression, and demonstrate that these anatomical peculiarities are actively developed from a common starting place as the singing male. Furthermore, they suggest *en grappe* NMJs (particularly outside of the motor endplate band region) may be the substrate by which the syrinx of the female zebra finch is incapable of making the complex and forceful motor movements necessary to produce song.

Future Directions

Use of Other Species of Birds, Particularly with Sexually Dimorphic Singing

Keeping with the tradition of numerous birdsong thesis discussions before mine, I will reiterate the extreme importance of translating this work to species other than the zebra finch. While the zebra finch is one of the most, if not the most, well-studied of the songbirds, they represent a particular adaptation in birdsong vocal learning. Female song is ancestral in songbirds, and currently present in at least three quarters of species, more recently lost in zebra finch females (Odom et al. 2014). It is tempting to attribute the female zebra finch syrinx and all its uniqueness relative to the male we've described here to be a substrate of evolutionary loss of behavior broadly. While I have presented a wealth of evidence here that indicates this may be the case, it is yet unclear whether this is a more broadly applicable anatomical substrate across even the songbirds, or a peculiarity of the zebra finch. Thus, it would be informative to repeat my experiments across a range of species in songbirds.

Strong inference could be drawn from other species with different degrees/kinds of sexual dimorphism in singing. There are other species where females also lack song (Bengalese finches, brown-headed cowbirds, etc.), where males and females are more equitable in their singing (European starlings, canaries), and where females might produce song as much as males, but of different types and therefore potentially different muscular and synaptic requirements for these varying behaviors (yellow-headed blackbirds, red-winged blackbirds). To date, there have been no well-described examples of songbird species in which females sing and males do not, but analysis of such a species would further elucidate the relative contribution of the anatomy described here in support of song suppression.

Indeed, recent work has taken a similar approach in pursuit of the peripheral anatomical differences that might support song in some species and sexes but not in others. Christensen and colleagues (Christen et al. 2017) analyzed the species described above and many others in the context of syrinx muscle fiber type, asking whether superfast fiber proportions in the syrinx are related to singing ability. Interestingly, they find a peculiarity in the zebra finch and the closely related Bengalese finch, that the proportion of superfast fibers is inverse between the sexes and positively correlated to song, while in other species these proportions are equitable and not sexually dimorphic. They conclude that, while superfast fiber composition is likely not the pan-species correlate of singing ability, it is potentially more related to the entirety of the vocal repertoire. Thus, the bias described here of *en grappe* NMJs towards weaker fast fibers might represent a peculiarity to some, but not all, songbird species where females do not sing. But overall, the evidence indicates that gross anatomical features such as syrinx weight, muscle fiber size, or even muscle fiber composition are likely tied to body size or are more species-specific than related to song production features, while the role of *en grappe* NMJs across species of songbirds is wholly

unknown. Further analysis of synaptic differences across these species would thus be informative to closing this gap.

Functional Studies of en grappe NMJs in this Species

It is worth noting that conclusions regarding *en grappe* NMJs being an anatomical substrate of song suppression hinge in part on descriptions of *en grappe* NMJs in other vertebrate models, where they are known to be weaker than the more prevalent *en plaque* NMJs. While the data presented here has contributed substantial insights into the structural characteristics of NMJs in male and female zebra finches, whether those kinds of functional differences are true in our model is unknown, and would be important to uncover in service of making stronger claims. By conducting detailed electrophysiological studies, insight can be gained into the firing kinetics, neurotransmitter release patterns, and postsynaptic response characteristics of *en grappe* NMJs. This can be accomplished *in vivo*, aided by either low concentrations (<5%) of BTX or 4di2Asp to identify NMJs in the muscles, and assessing properties of the *en plaque* NMJs in comparison to the *en grappe* NMJs both inside and outside of the MEB. Comparing these properties to those of *en plaque* NMJs offers a window into their unique functional attributes.

For instance, vertebrate *en plaque* NMJs are well-studied for their "all or none" responses - that is, every presynaptic action potential leads to a postsynaptic depolarization and muscle contraction. This also allows for quantal analysis in both types of NMJs, assessing the quantal content, size, and amplitude to reveal how these *en grappe* NMJs might mediate vesicle release, recycling, and postsynaptic responses relative to the more well-studied *en plaque* NMJs. Furthermore, electrophysiological studies would allow detailed analyses of facilitation and depression patterns, providing insight into synaptic plasticity mechanisms and release probability modulation, as well as better understanding their susceptibility to synaptic fatigue and recovery.

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Exploring these response properties in *en grappe* NMJs in the zebra finch model would reveal their reliability in translating neural input into muscle contraction, and further elucidate their role in motor control and behavioral variability.

Mapping of Innervating Motor Neurons

The innervation of the zebra finch syrinx is myotopic, that is, the more ventral syringeal muscles are innervated by more rostral motoneurons in nXIIts, and more dorsal muscles the more caudal portions of the motor pool (Vicario and Nottebohm 1988). This data was originally collected in adult males, and has not yet been done in females. Whether the same kind of innervation pattern is present in females broadly would be an interesting point of future study for at least a couple reasons.

First, adult females also have *en plaque* NMJs organized into an MEB as in males, but these NMJs make up only half of the total synapses in the adult female. Given that there are examples in my date of motoneurons that solely innervate *en plaque* or *en grappe* NMJs in the adult female, and adult males and females have similar numbers of innervating motoneurons in nXIIts, there is a distinct possibility that different motoneuron pools may not follow the same organizing principles in the brainstem. This is curious, given that there is no sex difference in electrophysiological properties of these neurons in adulthood (Roberts et al. 2007). Understanding the basic organization principles or these motoneurons, particularly in conjunction with differing innervation patterns, would be informative both to how the adult female innervation pattern ultimately develops, and to how central motor codes are ultimately translated into muscle movements and behavioral output. For example, motor unit size, i.e., the number of postsynaptic muscle fibers contacted by a single more axons, have been well documented for *en plaque* innervating motor neurons but there is little information on motor unit size for axons that

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predominantly innervate *en grappe* junctions. Motor unit sizes are the basis for understanding how force is produced in muscles via recruitment order, e.g., the Henneman size principle, and a better appreciation of motor unit sizes for *en grappe* motor axons would lead to a better understanding of how *en grappe* NMJs influence force production in the syrinx.

Second, the data reported here indicates that, to a first approximation, some motoneurons in the normal adult female syrinx innervate all combinations of NMJ classes, e.g., there are many examples of motoneurons innervating both *en grappe* and *en plaque* NMJs. These hybrid innervation patterns seem to only occur within the MEB, where both NMJ classes reside near each other in females, with the region outside the MEB having only *en grappe* NMJs, and seemingly motoneurons that only innervate this class. This conclusion is, of course, limited by the thin sections of our analysis, where only motoneuron axons within the volume can be traced, and it is possible that further along the axon would reveal these motoneurons also innervate *en plaque* NMJs at a different plane. Future study could take a classic tract-tracing approach in the adult female, injecting one color retrograde fluorescent tracer (i.e., GFP) into the MEB region, and a different tracer (i.e., RFP) into the region outside the MEB, and assess the organization of fluorescently labeled motor neurons in nXIIts. Whether there are motor neurons that solely innervate *en grappe* NMJs outside the MEB and follow the same myotopic organization as those that innervate within the MEB would be informative as to how motor signals are translated into muscle movements. There also exists the possibility that, much like the lack of organization into an MEB for *en grappe* NMJs in the female, there is a lack of organization in nXIIts of motoneurons innervating solely *en grappe* NMJs. Given that, in adult males, nXIIts is also innervated topographically by RA, this would provide an additional anatomical substrate by which female song might be suppressed.

Transcriptome Profiling of the Syrinx

The data reported here has provided valuable insights into the structural differences between male and female NMJs, as well as implications about sexually dimorphic developmental programs leading to these differences. Transcriptome profiling is a promising avenue for elucidating the molecular underpinnings of these differences. By examining gene expression patterns and regulatory networks specific to male and female NMJs, transcriptome profiling can unveil critical molecular players driving the observed structural variations. This approach holds the potential to uncover previously unexplored genes, signaling pathways, and molecular mechanisms responsible for the morphological distinctions I have identified.

It is readily apparent that male and female zebra finch syringes are different in adulthood, both in the data presented here and at differing levels of biology reported by others. Perhaps the most striking sex difference is the relative proportion of different classes of NMJs. The data reported here show primarily a single component of this synapse, the density and structure of postsynaptic ACh receptors. There are numerous other structural components and regulators of the NMJ system that could be informative to other structure-function relationships in support of our song suppression hypothesis (Sanes and Lichtman 1999). For instance, MuSK (Muscle-Specific Kinase) is a transmembrane tyrosine kinase receptor located on the postsynaptic membrane at vertebrate NMJs, and is a central player in the formation and maintenance of NMJs via promotion of AChR clustering. A similar role is played by Agrin, a large proteoglycan which interacts with MuSK. Cytoskeletal proteins such as Dystrophin and Utrophin play roles in connecting the cytoskeleton of the muscle fiber to the extracellular matrix, providing stability and structural integrity to the NMJ. There are numerous other Laminins and Collagens that make up the extracellular matrix and provide additional structural support to the NMJ, helping anchor to the

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surrounding muscle tissue. In addition, recent work has shown that the adult male zebra finch syrinx has all the molecular machinery necessary to synthesize its own steroid hormones locally, instead of relying solely on hormones secreted from the gonads (Schuppe 2022). Given the hormonal sensitivity of muscles broadly and the syrinx specifically, these local systems have the potential to be key direct effectors on muscle structure and maintenance, as well as behavior. Whether the same steroid synthetic machinery is present in female syringes broadly or at *en grappe* NMJs specifically is unknown, and would be essential knowledge to further refining functional theories.

Interestingly, my own cursory bioinformatics analyses have shown that on the Z chromosome (of which male birds have 2, while females have Z and W, "flipped" from most mammals), there are a large number of genes related to NMJ structure and function in vertebrates, including many of those mentioned above, as well as steroid-synthetic regulatory proteins, glial-derived neurotrophic factor, and even acetylcholine receptor subunits. Interestingly, this chromosome also includes genes whose dysfunction are associated with neuromuscular disorders, including *C9orf72* (one of the most common links to amyotrophic lateral sclerosis, also known as ALS or Lou Gehrig's disease; Smeyers et al. 2021), *MuSK* (associated with myasthenia gravis; Borges and Richman 2020), and *FKTN* (associated with Fukuyama congenital muscular dystrophy; Kobayashi et al. 1998). There are at least 143 genes highly associated with neuromuscular system structure and function, and at least 23 of these genes on the Z chromosome in the zebra finch, or approximately 16% of NMJ-related genes. The Z chromosome contains ~5% of all genes, and if all genes were randomly assorted throughout the genome, we would expect approximately 7 genes to appear on this chromosome, significantly less than found ($\chi^2 = 33.78$, $p<$1E-15). The chromosomal system in birds is also peculiar in the sense that there tends to be little

dosage compensation of these genes, i.e., males have 2 copies of Z-chromosome genes while females have 1, and males tend to have more than 1x the expression of those genes' transcripts (Itoh et al. 2010). Whether the same extends to NMJ-related genes on the Z chromosome has not been reported in detail, but is possible, if not probable. Thus, a more comprehensive and unbiased view of the molecular profile of male and female syringes would be informative to better understanding where and how these sex differences in syrinx NMJs arise, and how they might be supported by different molecular programs.

Transcriptome profiling would help identify genes and regulatory networks that exhibit differential expression between male and female zebra finch NMJs, potentially tying in functional roles of these genes in shaping NMJ structure. Transcriptomic analysis could unveil novel candidate genes that are responsible for the observed differences in synaptic morphology, motor endplate organization, and other structural features reported here. In particular, transcriptome profiling comparing the MEB to the region outside the MEB in females might uncover the unique molecular signals required for *en grappe* NMJs. Additional analyses across developmental ages might also help uncover the genes and pathways by which different types of putative pruning are occurring in this system. Such candidate genes and pathways could then guide functional studies, where specific molecules could be manipulated *in vivo* to validate their sexually dimorphic roles. With the current pace of advances in targeted gene manipulation methods, it is no small stretch to think that, after uncovering candidate genes and pathways that drive either male or female syrinx NMJ development, one might be able to manipulate a male syrinx to develop in a female manner (and vice versa), and observe how NMJ structure and singing behavior may change to further support the conclusion of *en grappe* NMJs being the substrate of song suppression.

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