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Development and regulation of breathing rhythms in embryonic and hatchling birds

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ABSTRACT

For many, if not all, air-breathing vertebrates, breathing-like movements begin while the embryo is still ensconced in an aqueous environment. This is because primordial regions of the CNS become spontaneously active during early gestation and then must functionally transform and specialize once air breathing commences. The degree to which the embryonic ventilatory control system is established and competent at birth is variable, however, even between different components of the respiratory system. Moreover, the embryological experiences of an individual can also affect the outcomes and responsiveness of ventilation to respiratory stimuli and these details have major clinical implications. The broad field of respiratory neurobiology still has much to learn about the ontogeny of breathing control systems, and the oviparity of birds provides a unique model to examine how early rhythms transform day-to-day as they become functional. This hybrid review and research article will highlight the contributions of birds to the study of breathing control during early development. We will detail what is currently known about the onset and maturation of respiratory rhythm generation and also provide novel data about the development of central chemosensitivity. Finally, we will review data regarding the development of peripheral afferent inputs during early development and discuss whole-animal reflex responsiveness to common respiratory stimuli, both chronic and acute, during the incubation period and following hatching.

1. Introduction

The avian respiratory system is a unique and experimentally tractable arrangement and thus birds are a useful and productive comparative model for the study of breathing among terrestrial vertebrates (Duncker, 1974; Scheid and Piiper, 2011a; Siefert, 1896). For a detailed review on the respiratory system in adult birds see Maina (2006) and Powell (2015) and for a review on the control of breathing in adult birds see Scheid and Piiper (2011b). As examples, bird-based models feature prominently in quantifying the physical details of gas exchange (Maina, 2002; West, 2009), as well as analyzing the functional and adaptive significance of air-flow through the rigid avian lung (Scheid, 1978; Scheid et al., 1972; Scheid and Piiper, 1989, 1972). Due to the distinctive anatomical structure of the avian lung and air-sac system, birds allowed for rigorous *in situ* experiments that employ continuous unidirectional ventilation, which makes possible the precise real-time study of the exchange of oxygen and carbon dioxide with the atmosphere and blood (Scheid et al., 1972; Banzett and Burger, 1977; Burger et al., 1979; Hempleman et al., 1983). The avian respiratory system has received significant attention in reference to their adaptations to high altitude (Ivy and Scott, 2015; Scott, 2011), diving physiology (Butler and Jones, 1997; Meir and Ponganis, 2009), and the coordination between breathing and body movements during locomotion (Funk et al., 1997, 1992a, 1992b; Klein and Codd, 2010). Additionally, birds possess unique and specialized intrapulmonary chemoreceptors (IPC) that remain a valuable model for understanding cellular mechanisms of CO_2 signal transduction in the context of breathing (Hempleman et al., 2006; Hempleman and Posner, 2004; Pilarski et al., 2009; Pilarski and Hempleman, 2007, 2006).

Birds are also a convenient and useful non-human vertebrate model to study breathing behavior during development, and that is the motivation for this document. Few other vertebrate taxa permit controlled experimental manipulations *in ovo* and *ex ovo* in concert with direct day-by-day investigations of the embryo. In addition to work examining cardiovascular physiology during incubation (for a review see Burggren et al. 2016), avian oviparity has provided many details of gas exchange through the eggshell as growth and metabolic rate increase toward hatching (A.R and Rahn, 1985; Mortola, 2009; Rahn and Paganelli, 1990). Studies examining the sensitivity and maturation of ventilatory reflexes at the onset of continuous air-breathing have also received a

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fair amount of attention (Menna and Mortola, 2003; Pilarski and Hempleman, 2007; Szdzuy and Mortola, 2008, 2007a, 2007b). Our primary goal here is to review, highlight and fill-in areas where our understanding of breathing-related ontogeny is incomplete. For example, understanding how breathing rhythms are organized and how they functionally differentiate prior to hatching is unknown. This is despite the continuous access to developing central and peripheral respiratory-related structures within the eggshell environment that bird embryos provide.

Specifically, this review will focus on past and current research related to the development and maturation of breathing control in birds. Although the majority of experimental data on this topic arise from the paranatal and postnatal period, *i.e.*, centered around the hatching sequence, we will also include the early embryonic period. We will proceed chronologically describing aspects of the development and maturation of putative central rhythm generating circuits that eventually organize into specialized breathing rhythms once continuous breathing begins. We also report for the first time how pH sensitivity develops in the isolated avian hindbrain. We will then detail the *in vitro* development of known peripheral breathing control elements followed by the *in vivo* inception of acute and chronic ventilatory reflexes in response to common respiratory stimuli both as the embryonic bird develops within the egg and following escape of the hard shell. We hope that our efforts serve to spotlight the significant opportunity that avian models provide to explore the development of breathing regulation and control, especially as transgenic approaches become more common (Agate et al., 2009; Liu et al., 2017). Bird models may be particularly effective in parsing out the subtle effects of developmental plasticity in response to early-life experience, and we will also review some of that information here.

2. Anatomical organization of the breathing-related avian hindbrain

It is presumed that in mature birds, as in mammals, autonomous breathing biorhythms for inspiration and expiration originate in the hindbrain. However, outside of work examining the anatomy of the songbird respiratory-vocal system (Ashmore et al., 2008; Schmidt et al., 2012), there is little known about the precise location of central breathing-related anatomy for avian rhythmogenesis (but also see Peek et al., 1975; Kubke et al., 2005; Wild et al., 2009; McLean et al., 2013; Pickett et al., 2018). Determining whether there is functional conservation of respiratory-related hindbrain structures among different vertebrate taxa would be informative and facilitate a common understanding of the rules of neural circuit assembly. This is because the avian respiratory system is structurally and functionally unique, including their expression of breathing rhythm and pattern. When birds ventilate, for example, both the inspiratory and expiratory phases are always active muscular events and the anatomical resting position of the thorax, furcula and sternum is midway between the inspiratory and expiratory phases (Bouverot, 1978; Siefert, 1896; Wilson, 2003).

There are limited examples of breathing-related and regionally-specific neural recordings from nuclei in the avian hindbrain, but data suggest a rostrocaudal column extending from a site near the spinomedullary border through the pons to the rostral extent of the mesencephalon. Fig. 1 compares details of the avian ventrolateral respiratory column and associated motor pathways with that of the mammal. Comparative data show that there is a potential for several spatially and functionally homologous respiratory nuclei between these taxa, despite their whole animal ventilatory differences. A number of studies, which used anesthetized but freely breathing adult zebra finch, describe the nucleus paraambiguus (PAm) and the nucleus retroambiguus (RAm) in the avian hindbrain as possible breathing-related rhythmic nuclei within the ventral respiratory column (Reinke and Wild, 1998, 1997; Sturdy et al., 2003). The PAm contains cells that discharge with or just prior to inspiration and may be a homologue to the mammalian preBötzinger area, but necessary and sufficiency tests of intrinsic rhythmogenesis have not been performed. Pickett et al. (2018) provided a demonstration that thick tissue slices centered on the exit of the glossopharyngeal rootlets can produce an inspiratory-like rhythm in the altricial zebra finch. This isolated slice (Fig. 1) captured the PAm, but probably not the RAm, and suggests a potential location for automatic inspiratory behavior. Data also indicate that the avian RAm contains cells that discharge in phase with expiratory movements (McLean et al., 2013; Sturdy et al., 2003). The RAm is likely to be developmentally homologous to the caudal ventral respiratory group (cVRG) in mammals, which contains expiratory pre-motor neurons, but is not rhythmogenic itself. Another candidate for expiratory rhythms is the avian nucleus infra-olivaris superior or IOS, which is possibly the same region as the parafacial respiratory group (pFRG)/retrotrapezoid nucleus (RTN) in mammals. While expiratory activity is present in the IOS (Reinke and Wild, 1997), this site has not been examined in isolation and awaits further tests of its function. Due to the avian breathing pattern, *i.e.*, the mandatory muscle activation for both phases, an interesting question is whether normal quiet breathing requires a separate expiratory rhythm generator or a single rhythm generator that creates both phases.

Another clue regarding the avian anatomy for central breathing rhythmogenesis comes from the similarities between genetic signaling of hindbrain networks in birds and mammals (Champagnat and Fortin, 1997). Although functional data are lacking, there is a presumed conservation of hindbrain nuclei in these taxa, despite considerable heterochrony in the timing of morphological characteristics, such as neural tube closure (Chatonnet et al., 2006). Beginning near embryonic day 2 (E2) in *Gallus gallus domesticus*, the neural epithelium in the hindbrain starts dividing into regular segments called rhombomeres (r) (Lumsden and Keynes, 1989). There are eight rhombomeres that develop in the chicken embryo and many mammals, and these compartments allow for little mixing of cells within their respective boundaries as they functionally differentiate (Lumsden, 1991). At this stage, rhombomeres are visible but transient structures that represent the future reticular formation and cranial nerve landscape. Embryonic birds studied *in ovo* are a common animal model for investigations aiming to understand the early anatomical features of the developing hindbrain because disruption of transcription factors that alter rhombomeric signaling leads to breathing issues in mammals (Caubit et al., 2010; Rose et al., 2009). Rhombomeric compartments are organized as a result of the expression of several gene families, such as Hox and Egr2/Krox20, and they can function for a time as independent developmental modules linking early control of genes to air-breathing behaviors (Coutinho et al., 2004). Fortin et al. (1999) demonstrated a role for r3/4 pairs in the development of early rhythmogenesis in the avian hindbrain using microsurgical grafting techniques, and these data are consistent with transgenic work targeting r3/4 development in mice. While the adult phenotype of r3/4 in the context of breathing is unknown in birds, r3/4 hindbrain segments seem to produce the parafacial respiratory group in mammals, which has implications for fetal breathing movements as well as expiratory rhythm generation in this taxa.

The focus of this type of genetic work is dominated by the mammalian viewpoint. Birds were merely a convenient developmental model as a representative vertebrate due to their embryonic accessibility, but they are currently overlooked as an animal model in the context of their own unique breathing behaviors. As mentioned above, rhythmogenesis for the two phases of adult ventilation are unique in birds, so testing the genetic control of putative respiratory nuclei in the brainstem of this group of animals will lead to a better understanding of central pattern and rhythm generating behaviors more broadly.

3. Rhythmic spontaneous neural activity in developing neural circuits

An individual bird egg is a self-sufficient aqueous compartment in

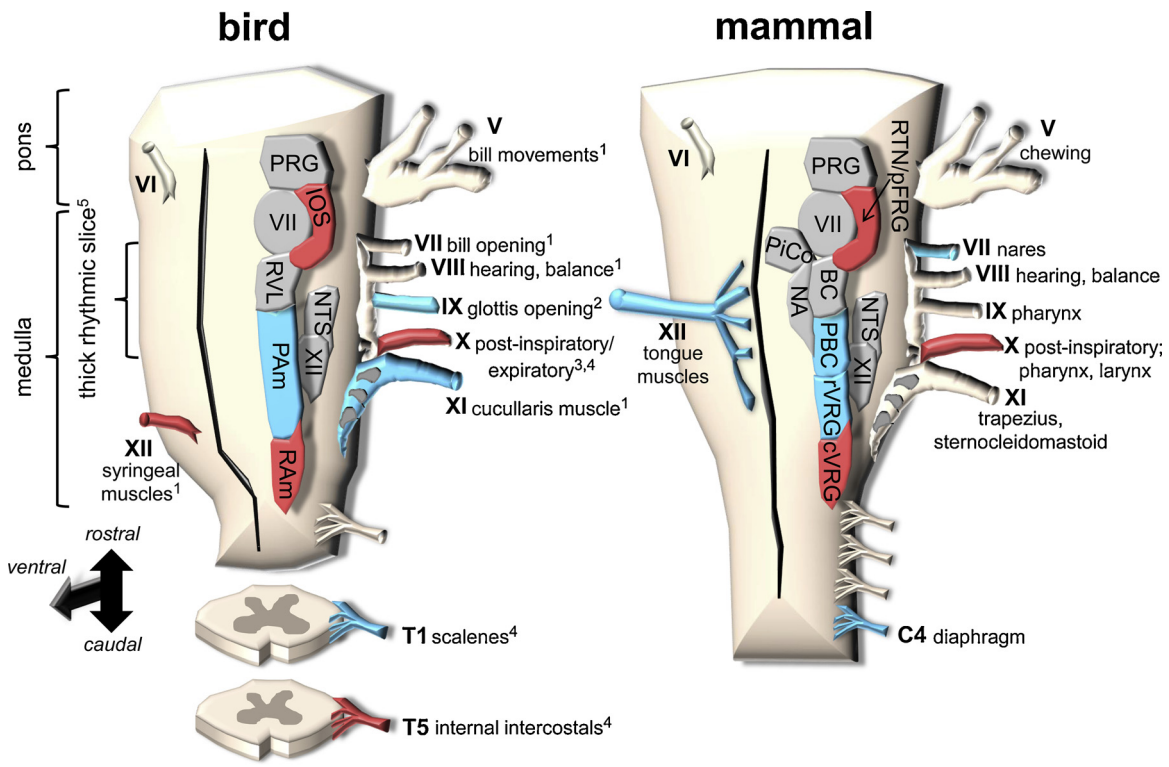

Fig. 1. Schematic diagram of the major ventrolateral hindbrain nuclei for respiratory rhythm generation, pattern and control in birds and mammals. This respiratory-related column originates in the pons rostrally and extends to the spinomedullary junction caudally. Ventral views show compartmental arrangements that have been described in both of these taxa. Respiratory nuclei that are thought to be functionally alike and possibly developmentally homologous appear as identical shapes and reside in similar locations in this representation. Red and blue shaded nuclei and transected cranial nerves indicate known inspiratory- and expiratory-related areas, respectively. In birds, the inspiratory phase of breathing coincides with neural activity in a longitudinal segment of the ventral-lateral medulla called the paraambiguus (PAm). In the mammal, a similarly positioned region of the medulla houses the Pre-Bötzinger Complex (PBC) and the rostral ventral respiratory group (rVRG). Although the bird does not have a muscular diaphragm, the relatively conserved layout of nuclei suggests that the unique breathing behaviors characteristic of these taxa may occur by alterations in activity or connectivity rather than major variations in hindbrain anatomy. The cranial and spinal nerves drawn laterally on the hindbrains allow for comparisons in motor output based on muscle targets and breathing phases, at least where data are known. The color of the nerve roots denotes inspiratory (blue) and expiratory (red) activity during normal quiet breathing (see the text and superscript reference #'s for more information). In mammals, expiratory activity is associated with elevated neural drive (*e.g.*, exercise), while birds actively produce an expiratory phase even at rest. Other major differences between these taxa include the innervation of the syrinx by the hypoglossal (XII) nerve in Aves (the syrinx is absent in mammals), and the potentially unique avian inspiratory motor out flows from the glossopharyngeal and spinal-accessory nerve. The similar topofunctional depiction of the IOS in birds and the RTN/pFRG in mammals is speculative, but see ref. 4 for discussion. Abbreviations: IOS, nucleus infra-olivarus superior; RVL, ventrolateral nucleus of the rostral medulla; PAm, nucleus parambiguus; NTS, nucleus of the solitary tract; Ram, nucleus retroambiguus; PRG, pontine respiratory group, PBC; Pre-Bötzinger Complex; BC, Bötzinger Complex; NA, nucleus ambiguus; rVRG, rostral ventral respiratory group; cVRG, caudal ventral respiratory group; PiCo, post-inspiratory complex; RTN/pFRG, retrotrapazoid nucleus/parafacial respiratory group. References: [1]King and McLelland, 1979; [2]Larsen and Goller, 2002; [3]Taylor et al., 1999; [4]Reinke and Wild, 1997; Wild et al., 2009; [5]Pickett et al., 2018.

which the calcium based shell creates a conductive barrier for the passage of CO_2, O_2 and water. As the embryo grows, there are gradual and inevitable physiochemical changes that may influence the *in ovo* environment and therefore developmental signaling during incubation (Fig. 2). However, few experiments have addressed the development of breathing behaviors in the context of physiological changes experienced by the encapsulated embryo. In this section, we discuss the maturational details of spontaneous respiratory rhythms emanating from the isolated brainstem where these data exist.

Rhythmic spontaneous neural activity (SNA) is a hallmark of the embryonic central nervous system, and this early form of bioelectricity results from large-scale depolarization waves that sweep across the developing nervous system (Blankenship and Feller, 2010; Momose-Sato and Sato, 2013; Moody and Bosma, 2005; Tabak et al., 2010). Experiments demonstrate that populations of motor neurons participate in SNA by locally synchronizing to produce intermittent depolarization events followed by a depression of network excitability (Tabak et al., 2001). This behavior also gives SNA a regular and consistent rhythmic quality that can be measured *via* classical electrophysiology, voltage sensitive dyes and calcium sensitive imaging (Hughes et al., 2009; Mochida et al., 2009; O'Donovan et al., 2005; Wang et al., 2009). It is important to note that the earliest form of rhythmic SNA seems to originate in the spinal cord. SNA then spreads anteriorly and posteriorly. However, most nervous system segments can also generate SNA when isolated during this time. As development proceeds, these generalized rhythmic depolarization events reorganize and differentiate into functional and localized circuits, some of which lose rhythmogenic independence, but the details of this transition are mostly unknown. Nevertheless, a substantial body of research supports the role of early spontaneous activity in neurogenesis and neuron survival, neuron migration, synapse formation, and other cellular processes (for a recent review see Landmesser, 2018). The proper formation of breathing control circuits in the hindbrain probably requires normal patterns of early spontaneous activity.

3.1. Development of rhythmic SNA in the avian hindbrain during incubation

Following rhombencephalon segmentation on E4, rhythmic SNA can be measured from the isolated avian hindbrain. Hindbrain SNA has been documented with extracellular motor axon recordings in the

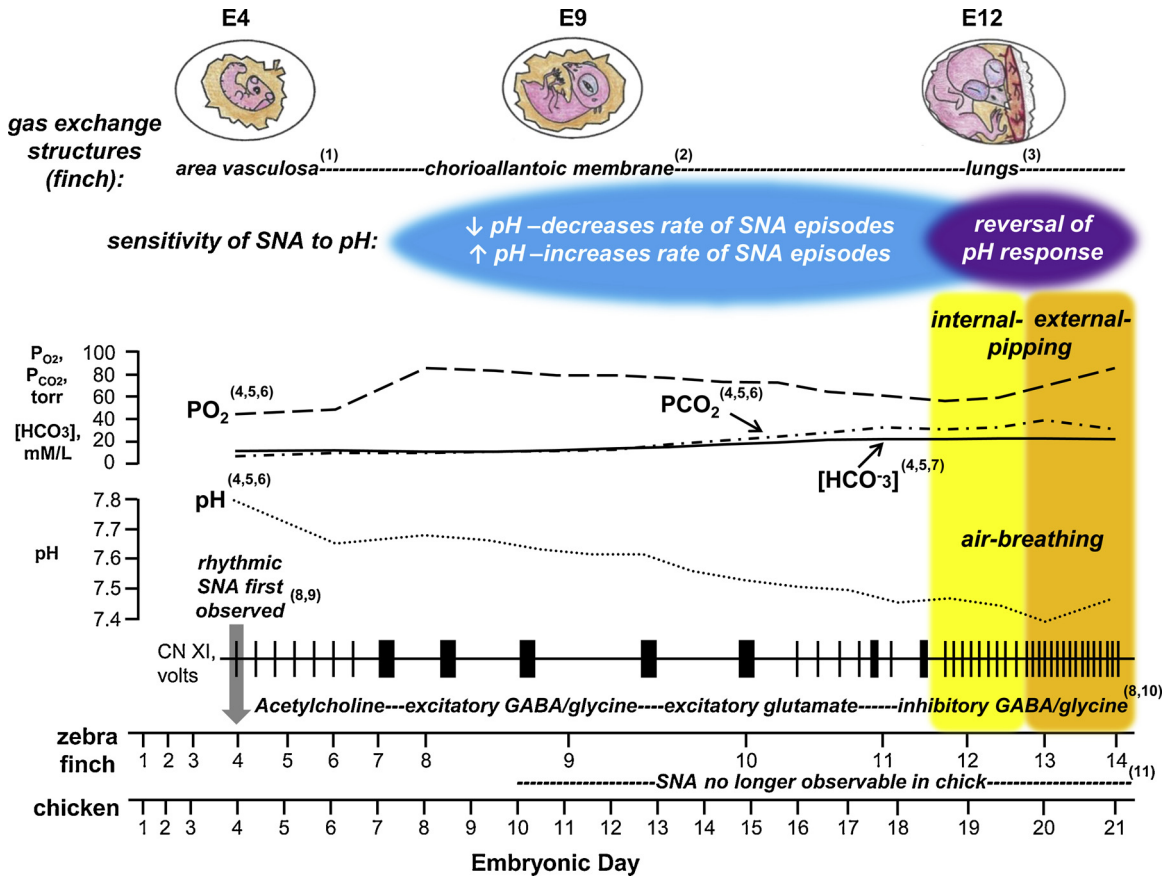

Fig. 2. A developmental time-line of the zebra finch and chicken incubation periods in the context of the developing respiratory system. From the early prenatal period through hatching, avian embryos experience changes in their physiochemical environment, resulting in embryos transitioning from purely diffusive gas exchange *via* the eggshell to convective pulmonary ventilation. Due to embryonic metabolism, the blood of vitelline and chorioallantoic arteries experience a decrease in pH (dotted line). This acidification is somewhat blunted by [HCO_3^-] (solid line) that compensates for the ramifications of rising PCO_2 (dashed and dotted line, -. -. -. -). The *in ovo* environment shifts during the transition to air breathing as PO_2 rises (dashed line) with the onset of internal and external pipping (yellow and orange shading). Further, rhythmic spontaneous neural activity (SNA) can be recorded from cranial nerves of the hindbrain that innervate accessory respiratory muscles in both chicken and zebra finch embryos on E4. The zebra finch allows a more complete picture of SNA from E4 through the onset of breathing-rhythms at hatching. Accordingly, we show the rhythmic SNA measured *via* cranial nerve XI, which highlights the changing pattern and complexity over chronological time (schematic drawing above time-lines; thicker lines indicate longer duration episodes). Rhythmic SNA transitions to an air-breathing motor outflow near E11-12 in the zebra finch and likely at E18-19 in the chicken. Interestingly, this transition to pulmonary ventilation coincides with a switch in the response to experimental acidification or alkalinization *in vitro* (blue and purple shading). Values for PO_2, PCO_2, [HCO_3^-], and pH were estimated based on data reported for chicken embryos. It is important to note that breathing rhythms *in vitro* have not been published from the later stages of chicken embryo development as the figure indicates. References: [1]Ackerman and Rahn, 1981, [2]Wangensteen and Rahn, 1970, [3]Chiba et al., 2002, [4]Tazawa et al., 1971, [5]Baumann and Meuer, 1992, [6]Meuer et al., 1989, [7]Meuer and Tietke, 1990, [8]Vincen-Brown et al., 2016a, [9]Fortin et al., 1994, [10]Pickett et al., 2018, [11]Chatonnet et al., 2002.

precocial chicken (Fortin et al., 1994, 1995, 1999; Vincen-Brown et al., 2016a), quail (unpublished personal observation), and the altricial zebra finch (Pickett et al., 2018; Vincen-Brown et al., 2016a). Fig. 2 shows the developmental time-line regarding a number of respiratory-related anatomical and physiological benchmarks during avian development within the eggshell. Hindbrain specific SNA appears *in vitro* as motor outflow from cranial nerves (CN) associated with the upper airway (Fig. 3), such as the glossopharyngeal (CN IX), vagus (CN X), and the hypoglossal nerves (CN XII) (Fortin et al., 1995; Vincen-Brown et al., 2016a, b; Pickett et al., 2018). More recently, studies in our laboratory found that the spinal-accessory nerve (CN XI) also carries rhythmic SNA (Fig. 3). Although the primary breathing mechanics are understood in adult birds, the accessory breathing muscles have received less attention. Exceptions come from Wild et al. (1981; 2009), Roberts et al. (2007) and Vicario and Nottebohm (1988) who have described the involvement of the hypoglossal motor nucleus in the context of breathing and vocalization. Data also show that the avian CN IX innervates the glottis and supports glottal opening during inspiration (Larsen and Goller, 2002).

The special visceral efferent projections that travel in the vagus nerve are more complicated. The vagus nerve seems to have axons that originate in the RAm (Wild et al., 2009) and therefore may transmit expiratory motor activity as is shown in Figs. 1 and 3C. The SVE vagal output also innervates the pharynx and palate (Taylor et al., 1999), but investigations are needed to understand the ventilatory role of the vagus motor function in birds. The vagal motor activity near hatching appears to be expiratory activity, perhaps a version of post-inspiratory activity, when compared to mammals (Manzke et al., 2009; Richter and Smith, 2014). Just like post-I neurons carried in the mammalian recurrent laryngeal nerve, the avian vagal motor outflow is actively inhibited by the inspiratory rhythm and seems to exhibit post-inhibitory rebound reminiscent of so-called handshake synchronization (see Fig. 3C and Wittmeier et al., 2008).

The spinal-accessory nerve innervates the cucullaris muscle, which is homologous to the trapezius and sternocleidomastoid muscles in mammals, and conveys regular rhythmic inspiratory activity in the bird *in vitro*. The CN XI motor output is synchronous with the inspiratory activity in CN IX (Fig. 3B), which may be an interesting difference in breathing mechanics when birds are compared to mammals. The mammalian SCM and trapezius are phasically active during ventilatory

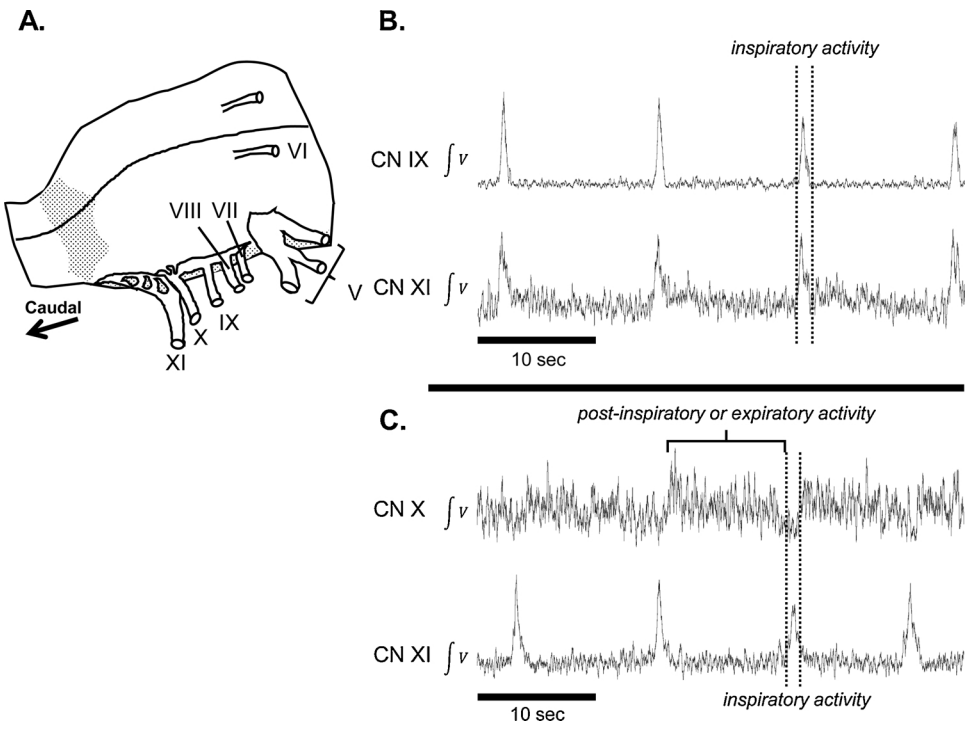

Fig. 3. Dual extracellular recordings of respiratory-related cranial nerve motor output in an E14 zebra finch embryonic hindbrain. **A.** Drawing of the E14 hindbrain preparation showing cranial nerves used in extracellular recording experiments. The anatomical borders of the preparation are shown as well. **B.** Representative rectified and integrated neural recordings of inspiratory cranial nerve motor output recorded simultaneously from CNs IX and XI. Traces show synchronous activation of the spinal accessory (CN XI) and glossopharyngeal nerves (CN IX). **C.** A dual recording from the same spinal accessory nerve rootlet (CN XI) as in B. and simultaneous activity from a rootlet of the vagus nerve (CN X). The vagus shows a decrementing pattern of activation following active inhibition during the spinal-accessory episode.

situations where high-respiratory drive is warranted (*e.g.*, exercise, gasping), but not during normal quiet breathing (Adams et al., 1989). In birds, the cucullaris muscle and its action has not been well-studied (but see Kierończyk et al., 2016), especially during breathing behaviors. Interestingly, all three of the cranial nerves innervating these muscles serve the nucleus ambiguus to some degree and exit the skull together through the jugular foramen, which leads to significant overlap and connectivity among these cranial nerves in the periphery.

When SNA begins, the *in ovo* compartment and the embryonic environment are relatively alkaline (Fig. 2). This is because the freshly laid egg first expels CO_2 accumulated while inside the maternal host and thus the overall alkalinity initially rises (Deeming and Denis, 2002). At the same time, the developing embryo is beginning to generate metabolic CO_2 and consuming O_2 which are exchanged with the ambient atmosphere through the extraembryonic membranes and the shell. The pH gradually begins to fall and the CO_2 content rises. For much of the first 1/3rd of incubation in the chicken the main respiratory organ is the area vasculosa of the yolk sac membrane. The CO_2 content of the venous outflow from the vitelline veins nearly doubles from E4 to E6, resulting in a pH drop from 8.0 to 7.89 (Meuer et al., 1989; Meuer and Tietke, 1990). This time period corresponds to the onset of the first phase of SNA in both chickens and zebra finch.

Given the physiochemical changes experienced by the embryo while *in ovo*, it is not surprising that SNA exhibits age-dependent alterations in rhythm and pattern, although the chemical signals for the steady network drive remains unclear (Fig. 2). In both the embryonic chicken and the zebra finch, despite their varied developmental strategies (precocial *vs*. altricial), rhythmic SNA first exhibits simple episodes of hindbrain activity at a rate of about 0.01 Hz. Rhythmic hindbrain SNA is initially facilitated by acetylcholine neurotransmission with an additional excitatory $GABA_A$ and/or glycine influence that adds to the overall excitation of neural activity (Vincen-Brown et al., 2016b). By recording from pairs of cranial nerves from chicken hindbrains on E4 through E10, Fortin and colleagues (1995) demonstrated that patterns of activation were similar and synchronized amongst the trigeminal, facial, glossopharyngeal, vagus, and hypoglossal motor nerves during this time period. These data support the hypothesis that early SNA is a network based wave that is relatively generic and undifferentiated and not simply a young version of the mature central pattern generator for breathing. The role of rhythmic hindbrain SNA at this chronological age is probably similar to spinal cord development. Landmesser (2018) reviewed the current understanding of activity-dependent mechanisms for synaptic target selection, motor neuron identity, and neurotransmitter phenotype in the spinal cord of *Gallus gallus*, but these details are unknown in the avian brainstem.

While the early SNA activity begins as single episodes from E4-6, from E7-10 the episodes of activity consist of complex trains of bursts within an individual depolarization event with longer durations and greater inter-episode intervals (Abadie et al., 2000; Vincen-Brown et al., 2016a). This change in the waveform of the rhythmic activity is associated with a transition from the early cholinergic and GABAergic excitatory drive to an ionotropic glutamatergic excitatory influence that persists until hatching. Interestingly, Vincen-Brown et al. (2016a) also showed that metabotropic glutamatergic (mGlu) influences are present during this mid-incubation time frame, but the role of mGlu receptors (mGluR) are unknown in this system. In neonatal mammals, data suggest a role for class I mGluR in network excitability and rhythm generation, especially when a high degree of electrical coupling is present (Nistri et al., 2006).

These alterations in pattern and timing of SNA suggest an ongoing process of organization in the neural circuits responsible for future breathing behaviors. It is also during this time that the respiratory function of the area vasculosa gives way to the developing allantois and chorion, which fuse to produce the chorioallantoic membrane. The chorioallantoic membrane covers the entire area inside of the inner shell membrane and supplies the permeable inner surface of the eggshell with blood vessel contact. This new respiratory organ is an attempt to meet the increasing demand for O_2. The embryonic environment also yields a worsening respiratory and metabolic acidosis, buffered in part by increased hydrogencarbonate concentration, which will continue until pulmonary ventilation begins around E12 in the zebra finch and E19 in the chicken (Fig. 2).

Following about 2/3^{rds} of avian incubation, data pertaining to the maturation of respiratory-related rhythmic motor behavior in the avian hindbrain are surprisingly limited. The last third of incubation is a critical time period that corresponds to the embryonic transition from

an aqueous environment to an aerial one in which pulmonary ventilation becomes continuous (Fig. 2). The birth of a bird, at least from the perspective of the newborn, is overtly similar to mammalian parturition, but it occurs over a comparatively extended time period. To date, one of the most complete records of the development and maturation of central breathing rhythmogenesis in birds comes from studies using the isolated hindbrain of the altricial zebra finch embryo, including the paranatal and early postnatal periods (Vincen-Brown et al., 2016a). Although neural recordings of early SNA are common in the chicken, rhythmic SNA does not seem to persist in this species beyond about E10-11 (Abadie et al., 2000), which precedes the onset of air-breathing behaviors. In fact, development of functional breathing-related central rhythm generation in this common experimental animal is mostly unknown, despite much work on features of whole animal respiratory control at hatching. The reasons behind the transient nature of rhythmic SNA in chickens is unclear but it may be related to the larger size and advanced maturity (*i.e.*, precociality) of the chicken compared to the zebra finch embryo. In other words, *the in vitro* tissue block of small altricial species may simply be more tolerant to prolonged periods in the recording chamber. Nonetheless, the resolution of this issue may yield some interesting general constraints of *in vitro* preparations, methodologies, and tissue viability along the altricial-precocial spectrum.

By E11-12 in the zebra finch, the hindbrain generated rhythm carried by CN IX and XI undergoes a significant change in its episode and burst characteristics, and this reflects the resting rate and duration of "newborn" inspiratory characteristics as observed in the whole animal. The timing of the increase in rhythmic SNA coincides with internal pipping into the air-cell in life and thus the onset of continuous pulmonary ventilation. The chorioallantoic and pulmonary ventilatory components both contribute to gas exchange during the paranatal period, but the added pulmonary respiratory element serves to alleviate a worsening respiratory acidosis (Fig. 2). The timing of internal pipping, as well as hatching, can be manipulated (earlier or later) by increasing or decreasing gas exchange through the shell over the air-cell space (Visschedijk, 1968), suggesting embryonic CO_2 and O_2 chemosensitivity (see below for more detail). It isn't until the hard eggshell is cracked by the bird (at external pipping) that the air-cell compartment's hypoxia and hypercapnia are fully relieved and adult blood-gas values are achieved. External pipping also triggers the elimination of the chorioallantoic respiratory component and the full participation of pulmonary gas exchange.

At the onset of functional air-breathing, rhythmic SNA from the glossopharyngeal and the spinal accessory branches can be described as respiratory-related motor outflows, instead of network derived rhythmic SNA. The cranial nerve inspiratory rhythms (CN IX and XI) continue to increase their pace from E12-14, with a sharp increase occurring from E12-13. Although glutamate is now the dominant excitatory neurotransmitter, *via* non-NMDA receptors (Vincen-Brown et al., 2016a), $GABA_A$ergic/glycinergic neurotransmission has transitioned from an excitatory to an inhibitory phenotype (Pickett et al., 2018). Fig. 2 also shows the chronological time when hindbrain pH sensitivity seems to switch its effect on breathing-related rhythms *in vitro* (see section 4 for more detail). It is unclear if the conversion to the adult-like pH ventilatory reflex is linked to the maturation of $GABA_A$ergic/glycinergic neurotransmission but the timing is intriguing and should inspire further work in this area.

3.2. Developmental plasticity in the avian hindbrain and spinal cord during the SNA period

The establishment of early biorhythms in birds provides a useful and tractable neural platform from which to examine mechanisms of activity-dependent development and plasticity in specific circuits. Synchronized network activity changes in its location and function over the course of embryonic development, and these well-defined embryonic transitions may serve as critical windows when mechanisms of motor development can be evaluated. The translational significance of understanding embryonic perturbations of the nervous system is immense, especially considering that pharmaceuticals and recreational drugs taken during pregnancy are an ongoing public health issue (Ross et al., 2015). For example, fetal breathing movements are known to be critical in the development of a healthy respiratory system and selective serotonin reuptake inhibitors are sometimes taken during pregnancy where they may have complicated effects on altering spontaneous neural activity, as well as prenatal and postnatal breathing function (Diav-Citrin and Ornoy, 2012; Marchocki et al., 2013; Mornson et al., 2005; Morrison et al., 2001). Another prominent example stems from the expanding legalization of marijuana. In some locales, it is now being marketed as an antiemetic during the first trimester of pregnancy, a time when teratogen exposure is most risky. Data show that the endocannabinoid receptor system regulates several aspects of nervous system development, such as cell proliferation, migration and lineage commitment (Ilyasov et al., 2018; Wenner, 2013). Interestingly, these cellular events are similar to the purported developmental roles of SNA itself.

In the context of birds, studying developmental plasticity has often focused on neural circuits and their associated motoneuron projections using the isolated chicken spinal cord model. The pioneering work of Landmesser and O'Donovan, 1984; O'Donovan and Landmesser, 1987, and Oppenheim et al., 1989 among others has provided a solid framework toward a mechanistic appreciation of rhythmic SNA and its effect on neuromuscular development. One of the main features of circuit development and target determination in the avian nervous system is the early expression of SNA induced calcium transients (Hughes et al., 2009; Wenner and O'Donovan, 2001), which can trigger secondary ionic currents (*e.g.,* I_A and $I_{K(Ca)}$) as well as pathways that regulate synaptic scaling and gene expression patterns in a broad array of model systems (Dolmetsch et al., 1997; Watt et al., 2000). It is now established that rhythmic SNA exhibits the phenomenon of homeostatic plasticity in which neural activity senses changes from a set-point and evokes various types of corrective behavior. For example, blocking rhythmic SNA by $GABA_A$ receptor antagonism, which is excitatory in the embryonic chicken spinal cord, results in a homeostatic recovery of excitability and rhythmogenesis (Gonzalez-Islas et al., 2009; Wenner, 2011). In another demonstration of the utility of the chicken spinal cord preparation to study early circuit maturation, Yoon et al. (2008) used a retroviral transgenic approach to add the inward rectifier potassium channel Kir2.1 in lumbar motor neurons that participate in rhythmic SNA *in vitro* and *in vivo*. Kir2.1 helps to establish the resting membrane potential and its knock-in phenotype dampens overall network excitability, intrinsic excitability and associated calcium activated currents.

Unfortunately, much less is known about the potential for developmental plasticity in the avian hindbrain. Using optical recording techniques in the chicken embryo (E5-9), Momose-Sato and Sato (2014) demonstrated that isolated brainstem networks are capable of homeostatic alterations in the form of increased excitability and recruitment to restore rhythmic SNA when initially detached from the spinal cord. The latter study also highlights the independence of rhythm generating mechanisms among different segments of the early chicken nervous system (see also Fortin et al., 1999, 1995). As these authors point out, studies of this type must consider a variety of location specific compensatory effects of the embryonic nervous tissue that can result from mechanical manipulations, which can occur separate from or in addition to local mechanisms of plasticity. In agreement with some of the spinal cord work (Chub and O'Donovan, 1998; Gonzalez-Islas et al., 2010; Wilhelm and Wenner, 2008), Vincen-Brown et al., 2016b showed that rhythmic SNA measured from breathing-related cranial nerves in the chicken embryo (E5-5.5) is under homeostatic control in that depressing activity by blocking acetylcholine receptors leads to an increase in overall network excitability and recovery of baseline rhythmic SNA. This study also confirmed the importance of excitatory GABAergic

neurotransmission for rapid compensatory changes to rhythmic network activity.

Still, a significant knowledge gap persists for assessing functional outcomes resulting from embryonic manipulations of rhythmic SNA. There are currently no studies that address acute or chronic aspects of neuroplasticity at chronological times that are near the onset of air-breathing. This is likely a result of the focused use of the precocial chicken as a model organism for this type of work. It is still an open question as to whether developmental abnormalities associated with excitability of primordial motor circuits in the avian hindbrain result in future functional maladies in breathing control.

4. *In vitro* development of central respiratory-related pH chemosensitivity in birds

The development of an embryo's gas exchange tissues and nervous system occur in a changing environment (Fig. 2). To date, rhythmic SNA in the avian hindbrain has largely been characterized in terms of rhythm, pattern, and major excitatory and inhibitory neurotransmitters. In a series of recent experiments, we asked the question: Does the electrochemical environment, specifically pH levels, drive rhythmic SNA? We reasoned that changes in the pattern and rhythm of SNA could be a reflection of the shifting environmental variables that surround the developing bird. Also, we wanted to assess when central pH chemosensitivity could influence the pacing of ventilation once air breathing starts.

In the intact paranatal bird, following internal pipping and the start of air-breathing, exposure to hypercapnia results in alterations in ventilation similar, albeit attenuated, to adults birds under the same conditions (see section 6.2 below). However, the spatial and temporal aspects of central pH (and CO_2) sensitivities of developing birds have received scant attention. Therefore, our laboratory has recently employed the isolated hindbrain of the altricial zebra finch embryo to test whether rhythmic SNA varies under acidic or alkaline conditions prior to and following the hatching sequence. In this section, our goal is to describe the prenatal and paranatal sensitivities of rhythmic SNA to acidic and alkaline extracellular pH. To our knowledge, these are the first insights into the development of central pH sensitivity in the embryonic avian hindbrain.

4.1. Brief methods and experimental design

A total of 64 zebra finch embryos were used in the following studies. All procedures were approved by the Institutional Animal Care and Use Committee of Idaho State University. Embryos were anesthetized and hindbrains isolated as previously described (Pickett et al., 2018; Vincen-Brown et al., 2016a). The *in vitro* hindbrain preparation was placed into a temperature-controlled chamber (27 ± 0.5 °C) and superfused with artificial cerebrospinal fluid (aCSF) that was formulated to mimic the extracellular environment *in ovo*. Control aCSF contained the following components (in mM): 120 NaCl, 26 $NaHCO_3$, 30 D-Glucose, 1 $MgCl_2$, 3 KCl, 1.25 NaH_2PO_4, 1 $CaCl_2$. Control aCSF was equilibrated with a gas mixture containing 95% O_2 and 5% CO_2 (pH 7.45). After hindbrain preparations equilibrated in the control aCSF for ~15 min, we recorded control spontaneous neural activity from CN XI using suction electrodes. At least 30 min of stable CN XI activity preceded any treatments. Electrical signals were amplified, filtered, and recorded to a personal computer. To accomplish low or high extracellular pH, we altered the sodium hydrogencarbonate concentration of the aCSF. These approaches were modeled after experiments investigating central pH chemosensitivity in neonatal rodent brainstem tissue *in vitro* (Eugenín et al., 2006; Kawai et al., 2006; Voipio and Ballanyi, 1997). We superfused each hindbrain with only one of the following aCSF solutions for 1 h, ***1.)*** normocapnic acidic: 13 mM $NaHCO_3$ pH 7.2; ***2.)*** normocapnic alkaline: 52 mM $NaHCO_3$ pH 7.8. Each preparation was tested with a single solution of either low or high pH aCSF. This approach provided an assessment of the effects of low or high pH on CN XI activity without any chance of earlier treatments influencing later activity. The average frequency of rhythmic SNA was calculated for the baseline and treatment periods of each experiment. We divided the average frequency during treatment by the average frequency during the control recording to normalize data to the percentage of control period frequency. This adjusted for slight differences in baseline rhythms between animals of the same age or within age groups. Based on the pattern of baseline rhythms during incubation (Pickett et al., 2018; Vincen-Brown et al., 2016a), we grouped embryos into three age bins, early: E4-6, middle: E7-11, and late: E12-14. We tested for significant effects of treatment, age, and treatment*age using a two-way analysis of variance (JMP 14). Tukey's HSD was used to examine significant differences of treatment effects between age groups.

4.2. Effects of normocapnic acidosis and normocapnic alkalosis on rhythmic SNA

Both normocapnic acidic (pH 7.2) and normocapnic alkaline (pH 7.8) aCSF influenced rhythmic SNA in the developing zebra finch hindbrain. Fig. 4 shows representative examples of CN XI activity recorded during the bath applications of normocapnic acidotic (panel A.) and normocapnic alkalotic (panel B.) aCSF on specific days within each age group. Comparing the responses side by side highlights the age-dependent differences in pH-induced changes to the frequency of rhythmic SNA. Fig. 5 A and B shows relative changes in the frequency of rhythmic SNA for each embryonic day in which rhythm is recordable during both treatments. Fig. 5, panels C. and D., show mean percent control frequency by age group during both treatments.

We found significant overall effects of treatment, age group, and treatment*age group ($P < 0.0001$, $P = 0.0019$, $P < 0.0001$, respectively) for normocapnic acidosis. For animals between the ages of E4-6, data show a non-significant decrease in the frequency of SNA episodes relative to control periods (74.6 ± 7.1%; $n = 7$; $P = 0.58$). It was our observation that E4-6 embryos showed an irrepressible rhythmic SNA and demonstrated some compensatory behavior during the hour-long treatments, which buffered the decrementing effect of the acidosis.

Embryos between the ages of E7-11 demonstrated a reduction in rhythmic SNA frequency compared to control conditions during normocapnic acidic treatment (64.3 ± 11.6%; $n = 17$; $P = 0.0037$), which differed from the late age group ($P < 0.0001$). Lowering extracellular pH typically results in a dampening of neuronal excitation in most non-respiratory neurons (Ruffin et al., 2014; Sinning and Hübner, 2013). Decreases in extracellular pH can reduce NMDA- and AMPA-receptor mediated currents (Giffard et al., 1990), and GABAergic neurotransmission can also be elevated by an acidic environment (Krishek et al., 1996). These alterations typically decrease excitability in non-respiratory neurons until pH disturbances can be mitigated by the activity of several pH-regulating transporters (*e.g.*, sodium-hydrogen exchangers). Putative pH and CO_2 sensitive regions in the mammalian breathing control circuitry possess ion channels and other cellular mechanisms to sense and respond to shifts in extra- and intracellular pH (Huckstepp and Dale, 2011). These features and the purported effects of pH-regulating transporters may contribute to the increased ventilatory efforts following acidification of the hindbrain breathing network (Putnam, 2001). Further, some pH-sensitive ion channels show changes in expression over the course of development, at least in the embryonic mouse hindbrain (Aller and Wisden, 2008) Thus, the slower rate of rhythmic SNA during low pH exposure may occur due to populations of immature neurons that lack specialization for transducing pH signals into a functional ventilatory response.

In contrast, normocapnic acidosis resulted in a significant increase in rhythmic, breathing-related SNA in embryos aged E12-14 when compared to control values (172.4 ± 22.6%; $n = 10$; $P = 0.02$). The change in E12-14 respiratory-related rhythmic motor outflow

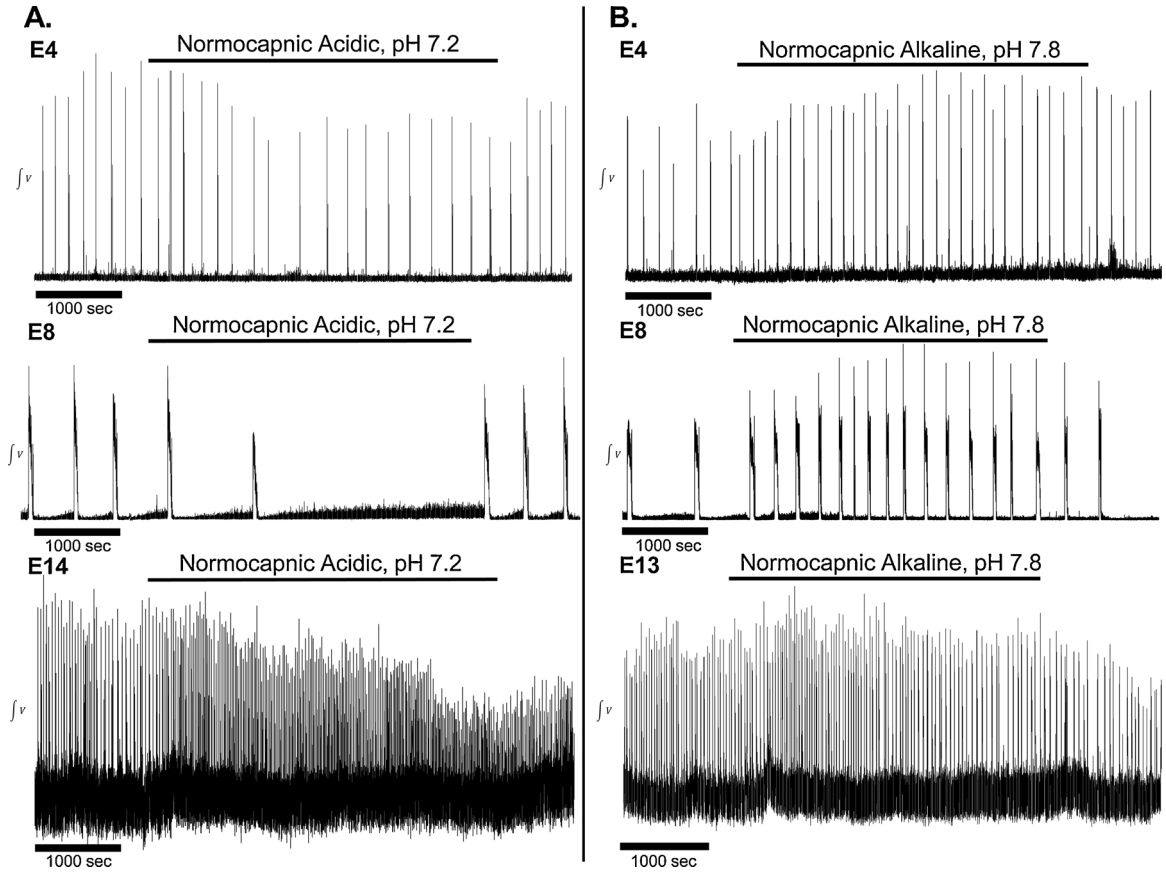

Fig. 4. Representative extracellular neural recordings of cranial nerve (CN) XI inspiratory motor output during either normocapnic acidic (low pH) or normocapnic alkaline (high pH) treatments. **A.** Both early and middle age groups show a slowing of rhythmic SNA frequency during acidic treatment. The late age groups show an increased frequency of rhythmic SNA during the same acidic treatment. **B.** Rhythmic SNA increases in frequency for early and middle age groups during alkaline treatment, while the late age groups show lower SNA frequencies in response to the same treatment.

significantly differed from both early and middle age groups ($P < 0.0001$, $P = 0.0002$, respectively). This observed switch in the response to acidity co-occurs with the transition to air-breathing which may represent an important step in maturation of the respiratory control system. Acidification in rodent hindbrain circuits for breathing tends to increase activity of the breathing network (Putnam, 2001). However, the exact locations, specific mechanisms, and maturity of central pH and CO_2 chemosensitivity remain an area of intense research.

Normocapnic alkalotic (pH 7.8) treatment resulted in the opposite pattern compared with normocapnic acidotic treatment (Fig. 4, panels B. and D.). Fig. 5, panels B. and D., show the relative changes in rhythmic SNA frequency for all ages between E4-14, as well as the pooled age group data. Embryos representing the early age group (E4-6) displayed little change in their rhythmic SNA during high pH (117 ± 17%, $n = 7$, $P = 0.98$). By E7-11, zebra finch embryos showed a significant increase in rhythmic SNA during alkaline treatment, which differed from the other two age groups (206 ± 25%, $n = 16$, $P < 0.0001$). In contrast to the earlier time periods, E12-14 air-breathing embryonic hindbrains tended to reduce their respiratory motor rhythm, although this did not reach the level of statistical significance (57 ± 11%, $n = 7$, $P = 0.6$). These results show that by the mid incubation period neural activity is excited by high pH, which is common in most non-respiratory neurons (Sinning and Hübner, 2013). Once embryos begin air-breathing at around E12, data suggest a switch in the pH response consistent with the onset of an adult-like corrective ventilatory response to metabolic alkalosis.

5. Development of breathing-related peripheral chemosensors and mechanosensors in birds

Although the role of peripheral chemosensors and mechanosensors in the ventilatory control of adult birds has received much attention (Burger et al., 1974; Fedde et al., 1974; Hempleman and Posner, 2004), relatively little is resolved about when these specialized structures first appear developmentally, as well as their functional chronology. The primary peripheral feedback to central respiratory control areas in birds are IPC, carotid bodies, and air sac mechanoreceptors. In this section, we briefly summarize the current knowledge on the developmental trajectory for each of these structures, *i.e.*, from their emergence to the onset of functionality in ventilatory respiratory control. Additionally, we compare what is known about their functional timing during development using acute chemoreflex data, as well as effects of chronic chemostimulation during early life which will be explained more in following sections.

5.1. IPC

Decades of research has led to the characterization of IPC in adult birds as vagally innervated chemosensors located within the parabronchial mantle that show an inverse relationship between firing characteristics and local levels of CO_2 (Hempleman and Posner, 2004; Peterson and Fedde, 1968). In other words, IPC firing rates slow as CO_2 levels increase. These sensors can monitor both oscillations in intrapulmonary CO_2 content as well as detect static or constant CO_2 levels in adult birds. To examine the maturation of IPC, a series of experiments were performed in which the inspired CO_2 was experimentally

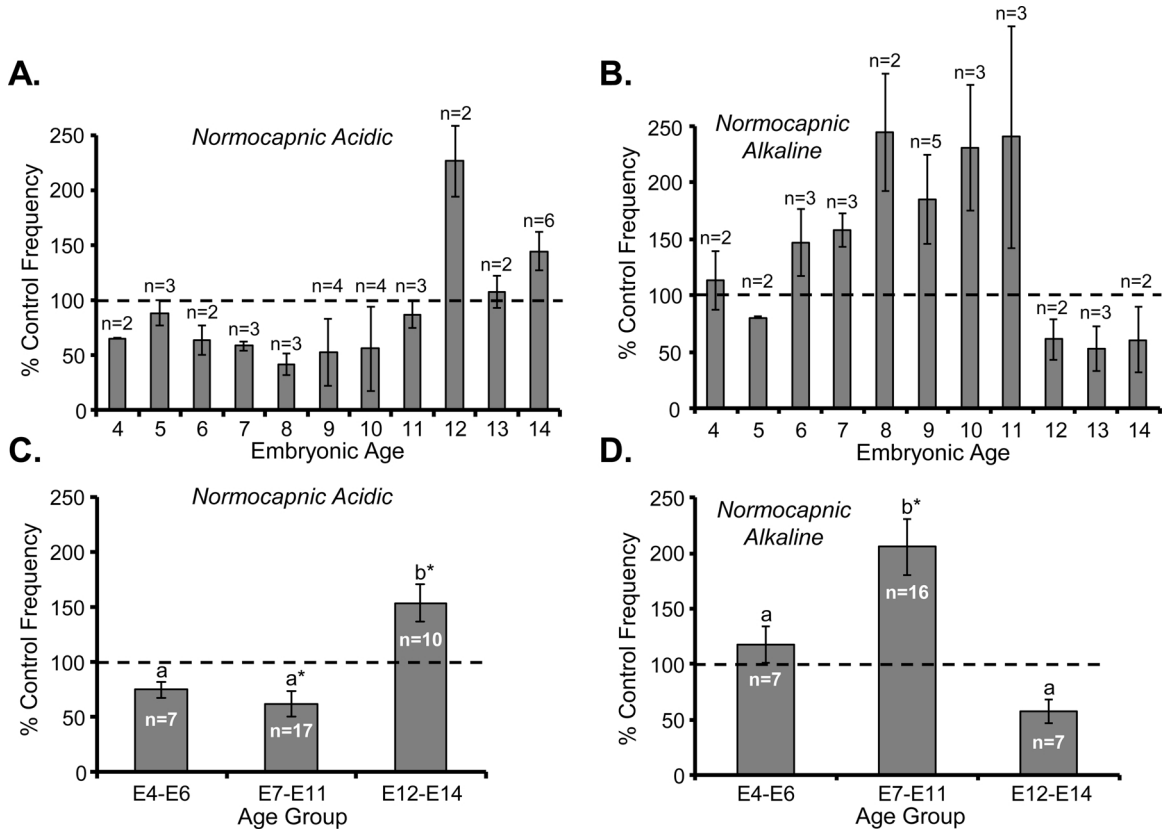

Fig. 5. Summary data for the effects of bath application of normocapnic acidic and normocapnic alkaline aCSF on CN XI activity in the zebra finch embryonic hindbrain. **A.** Mean frequency of rhythmic CN XI motor output as a percent of control frequency after 1 -h of low pH treatment. **B.** Mean frequency of rhythmic CN XI motor output as a percent of control frequency after 1 -h of high pH treatment. **C.** Mean frequency of CN XI activity as a percent of control frequency for developmental ages E4-6, E7-11, and E12-14 during low pH treatment. **D.** Mean frequency of CN XI activity as a percent of control frequency for developmental ages E4-6, E7-11, and E12-14 during high pH treatment. Error bars represent standard error of the mean (± SEM). Dashed line represents rhythmic SNA frequency under baseline conditions (*i.e.*, 100% of control frequency). Lower case letters (a, b) indicate significant differences between age groups ($P \leq 0.05$) and * indicates differences from control ($P \leq 0.05$).

Table 1
Ventilatory changes in developing birds exposed to acute changes in respiratory gases.

Species	Perturbation	Age[a]	Ventilation Change[b]	Selected References
Chicken *Gallus gallus domesticus*	Acute Hypercapnia	IP	2% CO_2:↑V̇E 4% CO_2: no change	Szdzuy and Mortola, 2007b
		EP	2% CO_2: ↑VT 5% CO_2: ↑VT; ↑V̇E	Menna and Mortola, 2003
		Hatchling	2% CO_2: ↑VT; ↑V̇E 4% CO_2: ↑VT; ↑V̇E	Mortola and Toro-Velasquez, 2014; Szdzuy and Mortola, 2008
	Acute Hypoxia	IP	15% O_2: no change 10% O_2: ↑V̇E	Szdzuy and Mortola, 2007b
		EP	15% O_2: ↑VT; ↑V̇E 10% O_2: ↑VT; ↑*f*	Menna and Mortola, 2003
		Hatchling	15% O_2: ↑VT; ↑V̇E 10% O_2: ↑VT; ↑V̇E	Mortola and Toro-Velasquez, 2014; Szdzuy and Mortola, 2008
	Acute Hyperoxia	EP	95% O_2: ↓ V̇E	Mortola, 2004
Muscovy Duck *Cairina moschata*	Acute Hypercapnia	Hatchling	2% CO_2:↑*f*, 4% CO_2: ↑VT ; ↑*f*;	Mortola and Toro-Velasquez, 2014 and unpublished data
	Acute Hypoxia	Hatchling	15% O_2: ↑*f*; 10% O_2: ↑*f*;	
Pekin Duck *Anas platyrhynchos domesticus*	Acute Hypercapnia	Hatchling	1.5% CO_2: no change 4% CO_2: ↑VT; ↑*f*; ↑V̇E	Sirsat and Dzialowski, 2016
Wedge-tailed Shearwater *Puffinus pacificus*	Acute Hypercapnia	IP	2-5% CO_2: no change 10% CO_2: ↑VT; ↓*f*	Pettit and Whittow, 1982; Sirsat and Dzialowski, 2016
		EP	2% CO_2: ↑VT 5% CO_2: ↑VT 10% CO_2: ↑VT	
		Hatchling	2% CO_2: ↑VT; ↓*f* 5% CO_2: ↑VT; ↑V̇E 10% CO_2: ↑VT; ↓*f*; ↑V̇E	
	Acute Hypoxia	IP	0% O_2: ↑VT; ↓*f*	
Red-**winged Blackbird** *Agelaius phoeniceus*	Acute Hypercapnia	Hatchling	2% CO_2: ↑VT; ↑V̇E 4% CO_2: ↑VT; ↑V̇E	Dzialowski et al., 2016
	Acute Hypoxia	Hatchling	15% O_2: no change 10% O_2: ↑VT; ↑V̇E	

[a] IP, internally pipped; EP, externally pipped; Hatchling, hatched from egg for 0–24 hours.
[b] V̇E, total ventilation; VT, tidal volume; f, breathing frequency per minute; ↑/↓, a statistically significant change in ventilation from the control group as reported in the referenced article.

manipulated in unidirectionally ventilated ducklings at prenatal, paranatal, and hatchling periods. These data suggest that IPC are able to sense abrupt changes in CO_2 within the airway at least 12 h prior to hatching or between the time the bird first begins to breathe within the air cell and when it breaks through the eggshell (Pilarski and Hempleman, 2007). Similar studies have not yet been performed in altricial bird species or other precocial species such as chicken or quail, therefore it is unclear whether the IPC develop with similar chronology

Table 2
Effects of chronic respiratory gas disturbances during early life on later ventilatory chemoreflexes.

Species	Chronic Perturbation	Exposure Time[a]	Chemoreflex Tested[b]	Age at Testing	Ventilation Change[c]	Selected References
Chicken *Gallus gallus domesticus*	Hypercapnia (4% CO_2)	Embryonic	HCVR	Hatchling	2% CO_2: ↓VT; ↓V̇E 4% CO_2: ↓VT; ↓V̇E	Szdzuy and Mortola, 2008
			HVR	Hatchling	15% O_2: ↓VT; ↓V̇E 10% O_2: ↓VT; ↓V̇E	
	Hypoxia (15% O_2)	Embryonic	HCVR	Hatchling	2% CO_2: ↓VT 4% CO_2: ↓VT; ↓V̇E	Szdzuy and Mortola, 2007a
			HVR	Hatchling	15% O_2: ↓*f*; ↓V̇E 10% O_2: ↓*f*; ↓V̇E	
	Hyperoxia (60% O_2)	Embryonic	HCVR	Hatchling	2% CO_2: ↓V̇E 4% CO_2: no change	Mortola, 2011
			HVR	Hatchling	15% O_2: no change 10% O_2: ↓V̇E	
Japanese Quail *Coturnix japonica*	Hypercapnia (2% CO_2)	Embryonic	HCVR	Adult (females)	6% CO_2: ↓V̇E	Bavis and Kilgore, 2001
			HCVR	Adult (males)	6% CO_2: no change	
	Hyperoxia (60% O_2)	Embryonic	HVR	Adult	11% O_2: no change	Bavis and Simons, 2008
		Post-hatch	HVR	Adult	11% O_2: no change	
		Embryonic + Post-hatch	HVR	Adult	11% O_2: ↓V̇E; ↓*f*	
Zebra Finch *Taeniopygia guttata*	Hypercapnia (2%-5% CO_2)	Embryonic	HCVR	Adult	6% CO_2: ↓V̇E	Williams and Kilgore, 1992
		Post-hatch	HCVR	Adult	6% CO_2: ↓V̇E	
		Embryonic + Post-hatch	HCVR	Adult	6% CO_2: ↓V̇E	
Bank Swallow[d] *Riparia riparia*	Hypercapnia (~2-5% CO_2) + Hypoxia (~17-14% O_2)	Embryonic + Post-hatch	HCVR	Nestling	3% CO_2: no change 4% CO_2: ↓VT 10% CO_2: ↓VT	Colby et al., 1987
			HVR	Nestling	15% O_2: no change 13% O_2: ↑*f*	

[a] Embryonic, before hatching; Post-hatch, after hatching.
[b] HCVR, hypercapnic ventilatory response; HVR, hypoxic ventilatory response.
[c] VE, total ventilation; VT, tidal volume; f, breathing frequency per minute; ↑/↓, a statistically significant change in ventilation from the control group as reported in the referenced article.
[d] Bank swallows nestlings were compared to adult swallows as the control group.

across altricial and precocial bird species. However, the responses of paranatal birds to the acute hypercapnia hints that IPC could be similarly functional in other precocial species (Menna and Mortola, 2003; Mortola and Toro-Velasquez, 2014; Sirsat and Dzialowski, 2016; Szdzuy and Mortola, 2008) and altricial species (Dzialowski et al., 2016; Pettit and Whittow, 1982), since all of these birds show changes in ventilation during acute CO_2 exposure where O_2 levels remained at a constant, normal level (see below and Table 1). Further, although IPC may not be fully functional for modifying ventilation until embryos initiate aerial breathing, the development of the IPC themselves, during the prenatal period, could be sensitive to abnormal incubation conditions occurring as a result of chronic high CO_2 exposure. Perhaps developing in an environment with heightened CO_2, which results in blunted hypercapnic ventilatory responses (HCVR) in chicken, quail, and zebra finch, alters normal development of IPC (Table 2). In adult birds, chronic hypercapnic exposure for twelve days increased IPC discharge and CO_2 sensitivity relative to control, which is consistent with a lower ventilatory response to elevated inspired CO_2 (Bebout and Hempleman, 1999). Nonetheless, the precise contribution of the IPC to the changes in ventilatory chemoreflexes following chronic hypercapnia will only be fully understood after experiments that somehow ablate or modify IPC development *in ovo* during chronic hypercapnic incubation. Genetic techniques or an *ex ovo* preparation may be ideal for accomplishing this feat.

5.2. Carotid bodies

Changes in O_2 concentration are primarily relayed to the CNS by the carotid body. Avian carotid body glomus cells detect hypoxia and respond by increasing the rate of action potential firing which is conveyed to respiratory control areas *via* the vagus nerve (Milsom and Burleson, 2007). Hypercapnia can also be detected by carotid bodies and triggers an increase in vagal afferent firing rate (Hempleman et al., 1992; Nye and Powell, 1984). The embryological development of carotid bodies has been followed using electron microscopy and histochemical labeling techniques (Fontaine, 1973; Hempleman and Warburton, 2013; Kameda, 1994). After migrating to their mature position, carotid bodies start to function just before hatching, which is inferred from ventilatory changes following acute perturbations in O_2 during internal pipping, external pipping, and hatchling phases (see Table 1 and Mortola and Toro-Velasquez, 2014; Pettit and Whittow, 1982; Szdzuy and Mortola, 2007b). Carotid bodies may play a role in the changes to ventilatory chemoreflexes following chronic hypoxic exposure during incubation. For example, exposure to hypoxia only resulted in blunted chemoreflexes when chick embryos were exposed during either the full 3-week incubation or the last week, during which carotid bodies become functional (Ferner and Mortola, 2009).

5.3. Mechanoreceptors

Ventilatory mechanoreceptors in birds are found in the air sac system (Kubke et al., 2004). Air sac mechanoreceptors detect the expansion and compression of these spaces during the phases of ventilation (Leitner and Roumy, 1974; Molony, 1974). Mechanoreceptors from avian air sacs provide vagal afferent input to ventilatory control centers *via* the nucleus of the solitary tract (NTS), but relatively little detail is known about this system in birds (but see Arends et al., 1988). Even less is known about the development and maturation of stretch sensitive air sac mechanoreceptors. We speculate that airway afferents are likely to remain nonfunctional until air-breathing begins at internal pipping and fluid is removed from the airway and air sac system. In mammals, including humans, vagal afferents, such as pulmonary stretch receptors, are somewhat functional, although not necessarily mature, at birth and can even evoke inspiratory and expiratory reflexes in the newborn (Fisher and Sant'Ambrogio, 1982; Frappell and MacFarlane, 2005; Hannam et al., 2001).

6. *In vivo* development of ventilatory chemosensitivity in birds

In a previous section, we showed data that breathing-related cranial nerve rhythms sped up in response to acidifying the environment of the zebra finch hindbrain near hatching (Fig. 5C). This finding suggests that functional central pH sensitivity may be in place by the time bird embryos begin to breathe air. IPC and carotid bodies also appear to be functional during the paranatal period. Accordingly, whole-animal ventilatory chemoreflexes are functional in late-stage bird embryos (paranatal) and hatchlings (postnatal) and contribute to the matching of breathing patterns with metabolic rate. To quantify ventilatory chemoreflex responses, studies tend to measure the hypercapnic ventilatory response (HCVR) and the hypoxic ventilatory response (HVR). The HCVR consists of an increase in ventilatory effort to eliminate excess CO_2 from tissues, and the HVR is initiated during periods of low systemic PO_2. Weak HCVR and HVR are linked to breathing conditions affecting human infants, highlighting the need to more clearly understand how these chemoreflexes arise developmentally (Carroll and Agarwal, 2010).

Similar to adult animals, breathing reflex development in newborns is studied by measuring changes in ventilation during hypercapnic or hypoxic gas exposures. Birds are a particularly useful vertebrate model to determine when ventilatory chemoreflexes first become functional since they exhibit a relatively slow transition to full-time pulmonary ventilation, first breathing within the confines of the hard shell prior to external pipping. While the domestic chicken is the most studied avian representative, a variety of other species allow us to consider the influence of additional factors, such as developmental strategy, *i.e.*, altricial and precocial birds, as well as life history effects (*e.g.*, burrow-nesting bank swallows) on ventilatory chemoreflex maturation. The inclusion of species-specific attributes may reveal divergent ventilation mechanisms that can overcome environmental stressors like hypercapnia or hypoxia. Here, our main goal is to consolidate data for the HCVR and HVR in mid-incubation (prenatal), near hatching (paranatal) and post-hatching (postnatal) birds such that new comparisons can be made.

6.1. Effects of hypercapnia and hypoxia on prenatal embryos in vivo

Although bird embryos experience increasingly acidic and hypercapnic conditions during incubation (Fig. 2), data suggest that chicken embryos are sensitive to hypercapnic and hypoxic stimuli even before the onset of pulmonary ventilation (Windle and Barcroft, 1938). Through a window in the chicken eggshell, uncoordinated contractions of respiratory muscles are visible as early as E13 and rhythmic breath-like movements begin on E14 (Windle and Barcroft, 1938). To investigate the sensitivity of embryonic "breathing" activity to hypercapnia and hypoxia, windowed-eggs were placed in enclosed chambers containing a variety of hypercapnic and hypoxic gas mixtures. As CO_2 levels increased, chicken embryos that had not yet initiated aerial ventilation showed overall changes in their body movements. At slightly elevated CO_2 concentrations (0.8–1.6%), E13-20 embryos demonstrated pecking motions and possibly fetal breathing movements. At higher CO_2 concentrations (5–10%), embryos of the same age appeared to gasp, struggle, and stop moving except to take deeper "breaths." Similar body and breathing movements were evoked by hypoxia (12% O_2) except that the onset of squirming and wiggling required a stronger stimulus (10–12% O_2) until breathing movements eventually ceased at O_2 levels < 10%. While these data suggest that altered respiratory gases impact whole body movements and breathing muscle contractions in the prenatal chicken, it may be difficult to distinguish whether hypercapnia or hypoxia act directly on fetal breathing movements or if the response is more global. It is possible that these gaseous stressors act more generally on the developing nervous system and modify gross embryonic behavior such as stimulating muscle contractions and *in ovo* motility. Thus, in chicken embryos that are too young to breathe air, hypercapnia or hypoxia may not evoke a ventilatory chemoreflex *per se*, but rather, change the overall excitability of the embryonic nervous system.

6.2. Ventilatory chemoreflexes in paranatal embryos in vivo

Studies of the HCVR and HVR in wedge-tailed shearwater and chicken embryos represent most of our knowledge of whole-animal ventilatory reflexes during internal and external pipping. These embryos are situated toward the opposite ends of the altricial to precocial spectrum with the shearwater exhibiting a greater need for parental care and chickens more independent at hatching. Thus, differences in the emergence of sensitivity to CO_2 and O_2 may be related to the degree at which peripheral and central chemosensory areas are functional when air-breathing begins.

Table 1 summarizes changes in ventilation that characterize the HCVR and HVR for the chicken and wedge-tailed shearwater during internal and external pipping. In general, data show that the HCVR and HVR are functional by the time pulmonary ventilation starts in chicken and wedge-tailed shearwaters (Menna and Mortola, 2003; Pettit and Whittow, 1982; Szdzuy and Mortola, 2007b). Both of these birds seem to respond to increased CO_2 or decreased O_2 primarily by increasing their tidal volumes. Some occasional modifications in rate of breathing are observed in the HCVR of internally pipped shearwater embryos as well as in the HVR of chicken embryos with shearwaters tending to slow their breathing and chickens breathing more rapidly. For the chicken embryo, increased total ventilation occurs under hypercapnic and hypoxic conditions as a result of the enhanced tidal volumes described above. In contrast, no significant changes in total ventilation were reported for shearwater embryos.

A careful comparison of the HCVR of chicken and shearwater embryos at internal pipping and external pipping highlights differences that may be related to the maturational timing and function of respiratory-related CO_2 chemoreception. In chicken embryos, internal pipping birds increase their total ventilation when breathing the weaker level of two CO_2 stimuli (2%) (Szdzuy and Mortola, 2007b). In contrast, the internal pipping shearwater embryos only demonstrate significant ventilatory changes when exposed to the highest CO_2 level (10%), which suggests they may be less sensitive to CO_2 during internal pipping than chicken embryos. However, these differences in chemosensitivity could also be related to different endogenous CO_2 levels within the egg (contributing to less responsiveness to 2–5% CO_2) or even differences in methodology. In the shearwater study, birds were exposed to increasing CO_2 levels sequentially while in the chicken embryo experiments birds were exposed to only one gas mixture in random order to minimize chances of carry-over effects confounding results (Menna and Mortola, 2003). By the time both birds reach external pipping, the differences in chemosensitivity are no longer present, *i.e.,* both species show increased ventilation to all levels of CO_2 stimuli. It is important to note that external pipping in the shearwater embryo refers to the formation of a pip-hole in the eggshell rather than the initial star fracture which precedes internal pipping in this species (Pettit and Whittow, 1982). Also, we did not attempt to compare the trajectory of O_2 sensitivity from internal to external pipping since the shearwater embryos were subjected to one O_2 level (anoxia) and data were only available for the internal pipping stage. Acute hyperoxia also alters breathing in externally pipping *G. gallus* mainly by lowering total ventilation (Mortola, 2004). However, it is currently unknown whether altricial birds show similar reductions in ventilation under high O_2 conditions.

6.3. Ventilatory chemoreflexes in hatchlings in vivo

Compared to the internal pipping stage of avian development, more data are available for the maturation of the HCVR and HVR once birds hatch into the ambient atmosphere (Dzialowski et al., 2016; Mortola

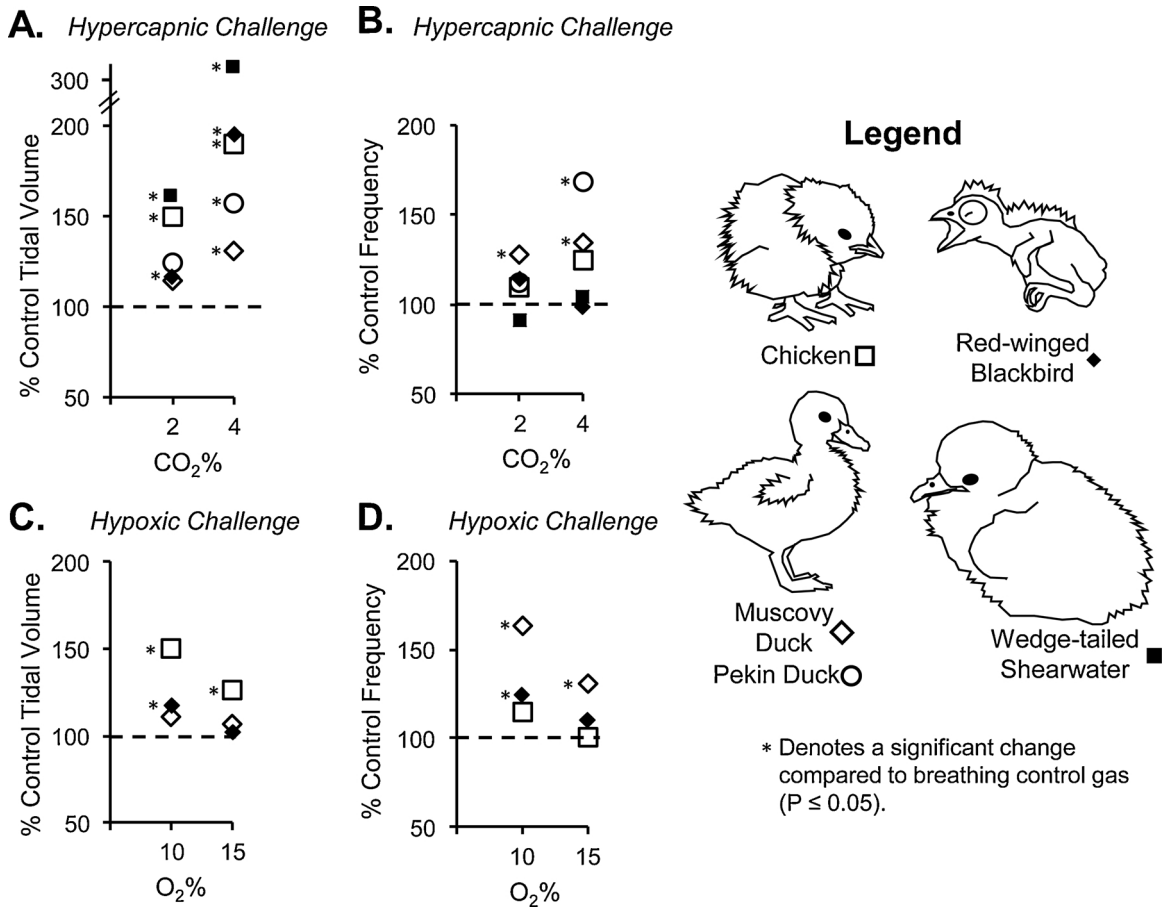

Fig. 6. Mean changes in tidal volume and breathing frequency reported for the hypercapnic and hypoxic ventilatory responses in altricial and precocial hatchlings of different species. **A.** Tidal volume (% of control) was above control levels for all birds exposed to the most severe level of hypercapnia (4%) and most birds exposed to 2% CO_2. **B.** Frequency of breathing (% of control) was above control levels for the ducks during hypercapnic exposure. **C.** Tidal volume (% of control) was above control levels for the chicken and for the red-winged blackbird at 10% O_2. **D.** Frequency of breathing (% of control) was raised compared to control levels in the Muscovy duck and the red-winged blackbird at 10% O_2. Mean values were computed from the data of Dzialowski et al. (2016), Mortola and Toro-Velasquez (2014), Pettit and Whittow (1982), Sirsat and Dzialowski (2016), Szdzuy and Mortola (2008). Significant differences from control tidal volume and frequency (dashed line = 100% of control ventilatory parameter) are indicated by an asterisk (*). Open symbols = precocial birds and closed symbols = altricial birds.

and Toro-Velasquez, 2014; Pettit and Whittow, 1982; Sirsat and Dzialowski, 2016). Past studies have collected data from a variety of species including the domestic chicken, Muscovy and Pekin ducks, wedge-tailed shearwater, and red-winged blackbird. Table 1 provides a summary of the HCVR and HVR for these species with the three precocial species (*i.e.,* chicken and ducks) listed above the two altricial species (*i.e.,* shearwater and red-winged blackbird). Overall, hatchlings display significant changes in either depth (*i.e.*, tidal volume) or frequency of breathing during acute hypercapnia or hypoxia, which contribute to significant changes in total ventilation in some cases.

To assess whether altricial and precocial bird species differ in terms of the magnitude of HCVR and HVR, we plotted tidal volume and breathing frequency relative to control for hatchlings of two altricial species and three precocial species using available data from the literature (Fig. 6). Without access to the original data, it was not possible to directly compare the effects of treatments between species. We presented these data in this manner to identify possible patterns between altricial and precocial species. Both altricial hatchlings (solid symbols) and precocial hatchlings (open symbols) exhibit elevated tidal volumes when breathing a high CO_2 gas mixture (Fig. 6A). Chicken, Pekin duck, shearwater, and blackbird hatchlings show significantly elevated total ventilation likely due in part to increased tidal volume during acute hypercapnia (Table 1). Data suggest that altricial species show a higher increase in tidal volume with high CO_2 concentrations compared to precocial birds. Also, the Muscovy duck and Pekin duck, showed significant increases in breathing frequency under high CO_2 conditions, while both altricial species and the chicken do not change their breathing rates (Fig. 6B).

There were fewer studies reporting the HVR for hatchlings, therefore similar plots of relative changes in tidal volume and frequency show fewer data points (Fig. 6C, D). Further, it is difficult to draw conclusions about the impacts of developmental strategy on the HVR since one altricial species was tested in hypoxic conditions comparable to studies of precocial species at hatching. For tidal volume, both the altricial red-winged blackbird and chicken showed significant increases when breathing the lowest O_2 gas mixture. Total ventilation subsequently increased for chicken hatchlings exposed to 15 or 10% O_2 and for blackbird hatchlings exposed to 10% O_2, as well (Table 1). For breathing frequency, the red-winged blackbird and Muscovy duck showed increases at the most severe level of hypoxia. From these data, it is apparent that the precocial species showed ventilatory alterations when breathing either level of hypoxia, while the altricial species only increased their tidal volume and frequency during the most severe hypoxic stimulus. Although additional studies are warranted, currently available data suggest that altricial and precocial hatchling birds employ functional carotid bodies since they both show modifications in breathing in response to acute hypoxia (Dzialowski et al., 2016; Ferner and Mortola, 2009).

To date, we are unaware of any studies measuring the effects of intermittent hypoxia on the development of breathing behavior in

birds. Intermittent hypoxia is a well-represented topic in the mammalian breathing literature as a model of the periodic hypoxic episodes that occur during sleep disordered breathing and obstructive sleep apnea, which, in pregnant women, can lead to poor outcomes for infants (Louis et al., 2014). Birds could be well-suited to studying the impacts of intermittent hypoxia on breathing control development since the embryos are accessible and lack confounding influences from a maternal host.

6.4. Effects of chronic embryonic exposure to altered O_2 and CO_2 and the development of postnatal ventilatory reflexes

Since both paranatal and postnatal birds exhibit ventilatory responses to acute hypercapnia and hypoxia, as well as increased embryonic motility prior to continuous ventilation, the capacity to sense and respond to respiratory stimuli is present during the prenatal period. However, it is unclear as to how and to what degree long-term alterations in the normal gaseous environment determines ventilatory reflex parameters. Questions about the magnitude, timing and future impact of developmental plasticity during early life are relatively new, especially from the perspective of breathing control. This is because the maturation of the respiratory system and its regulation was presumed to be predetermined *via* a genetic plan. Moreover, the number of different models, developmental stages, and methodological parameters are difficult to compare. Nevertheless, in this section we attempt to highlight two lightly utilized experimental paradigms (laboratory based *vs*. "natural" experiments), which may shed some light on the contributions of environmental cues and/or inherited factors to the development and maturation of breathing control in birds.

Remarkably, laboratory studies examining whether chemoreflex development is experience-dependent in freely behaving birds have been performed in just two open-nesting precocial species (chicken and quail) and one non-burrowing altricial bird species (zebra finch) (Bavis and Kilgore, 2001; Ferner and Mortola, 2009; Szdzuy and Mortola, 2008; Williams and Kilgore, 1992). Table 2 provides an overview of this group following prolonged respiratory gas manipulations during incubation. In chickens, hatchlings exposed to chronic hypercapnia or hypoxia during incubation showed decreased tidal volume and total ventilation when their HCVR or HVR were tested compared with control hatchlings. Surprisingly, quail do not consistently show blunted HCVR and HVR responses following either hypercapnic or hyperoxic exposure during incubation, at least not as an adult. Rather, sex and other inherited factors seem to play a role since only females and quail from specific hatcheries show reduced total ventilation ($\dot{V}E$) when their HCVR or HVR are quantified (Bavis and Kilgore, 2001; Bavis and Simons, 2008).

The effects of sustained hypercapnia on the zebra finch HCVR currently represent the only data for altricial bird species (Williams and Kilgore, 1992). This study tested whether exposure during incubation had a persistent effect on the HCVR in adult birds. Similar to the quail studies, the HCVR was measured in adults after they had been exposed to increased CO_2 during one of three time periods during incubation (Table 2). In adult zebra finch, as in quail, the hypercapnic experience during the embryonic, post-hatch, or both embryonic and post-hatch periods resulted in significantly blunted HCVRs. Overall, these data show that embryonic (and sometimes post-hatch) exposures to altered respiratory stimuli lead to persistent changes in the normal ventilatory responses associated with CO_2 and O_2.

In addition to chronic high CO_2 or low O_2 exposure during incubation, sustained high O_2 or hyperoxia can alter a bird's baseline ventilation and the magnitude of ventilatory chemoreflex responses (Bavis and Simons, 2008; Mortola, 2011). Adult quail show changes in baseline ventilation (*i.e.,* higher tidal volume) after hyperoxic exposure during the prenatal period (Bavis and Simons, 2008). Similar degrees of sustained hyperoxia during the last week of incubation in chicken embryos resulted in hatchling HCVRs and HVRs with significantly lower increases in total ventilation (Mortola, 2011). To our knowledge, it is unknown if chronic hyperoxic conditions exert similar influences on breathing in altricial birds.

To further explore the pattern associated with these results, we searched for avian examples of "natural" experiments that would result in chronic exposures to abnormal respiratory gas concentrations during incubation. We found a single study using nesting bank swallows as a comparison (Colby et al., 1987). The eggs of bank swallows naturally experience hypoxic (~17% O_2) and hypercapnic (~3% CO_2) conditions within their nest chambers (Birchard and Kilgore, 1980). Similar to the open-nesting bird species described above, ~2-week-old bank swallow nestlings showed an intact HCVR and HVR when evoked with acute respiratory gas disturbances. Colby et al. (1987) reported these ventilatory changes in juveniles as "blunted" compared with adults (Table 2). However, this comparison is fundamentally different than those in the experimental studies listed above. We can assume that the adult bank swallows experienced the same hypoxic and hypercapnic conditions during their own "chickhood" and may also have relatively blunted respiratory chemoreflexes (see below).

In an attempt to more clearly understand the consequences of a natural, burrow-experience on ventilatory control development, we roughly compared the HCVR and HVR of nestling bank swallows with open-nesting, hypercapnia/hypoxia-naïve, red-winged blackbird nestlings from the literature. Both altricial species showed significant increases in tidal volume and total ventilation when breathing 2–4% CO_2 gas (Colby et al., 1987; Dzialowski et al., 2016). While full datasets and matching gas concentrations are necessary for proper statistical comparisons, the swallows' HCVR seem to be reduced in magnitude compared with the blackbird. When both birds breathe 4 or 4.5% CO_2 gas, and their breathing parameters are normalized to percentage of their control air values, the swallows increased tidal volume to ~152 ± 12% of control while the blackbirds raised tidal volume to ~262% of control. Similarly, juvenile swallows and blackbirds display increased breathing frequency and total ventilation upon exposure to acute hypoxia (*i.e.,* 10 or 13% O_2) (Colby et al., 1987; Dzialowski et al., 2016). A similar normalization of breathing data for the HVR reveals an increase in breathing frequency of 136 ± 9% of control in the swallows while the blackbirds show an increase of ~203% of control frequency. Together, these data suggest that a natural burrow environment influences the control of breathing in young birds.

Given the bank swallow nestlings' hypoxic and hypercapnic surroundings, one explanatory hypothesis for these results is a suite of phylogenetic adaptations to alleviate or simply not respond to acid-base disturbances when faced with high ambient PCO_2 and low PO_2. Bank swallows' blunted ventilatory chemoreflexes may be evolutionarily shaped such that even if their eggs were incubated in an open nest environment, the young would still display lower increases in ventilation upon hypercapnic exposure, but this experiment has not been done.

To begin to address this question, it seems confounding to compare the bank swallow nestlings to adults of the same species (as discussed above). Instead, we took existing data for the adult bank swallows and compared those to the control zebra finch adult data and the adult zebra finch data from the previously described chronic exposure study (Colby et al., 1987; Williams and Kilgore, 1992). Again, these comparisons are imperfect and serve to point out opportunities for specifically designed experiments in the future. Not surprisingly, adult bank swallows showed somewhat smaller increases in tidal volume and total ventilation compared to control zebra finch adults during acute exposure to the same level (6%) of CO_2. Bank swallow adults showed an increase in tidal volume of 217 +/- 21% of their tidal volume while breathing room air. In contrast, control zebra finch adults showed a slightly higher increase in their tidal volume of ~231% of their control tidal volume. Further, the adult bank swallow HCVR was still blunted compared with adult zebra finch exposed to chronic high CO_2 during embryonic and post-hatch periods (*i.e.,* total ventilation increased to

~278% of control for finch *vs* 251 +/- 33% of control for the swallows). We were unable to compare the HVR for these two species since zebra finch were not tested with acute hypoxia, to our knowledge. Despite the need for experiments explicitly examining the role of an evolutionary history of enclosed nesting on the development of ventilatory control, these rough comparisons provide some clues that a burrow experience may not be equivalent to an experimentally induced gas perturbation during the incubation of a non-burrowing bird species. The reported reduced sensitivity to high CO_2 in bank swallow nestlings suggests that the development of peripheral CO_2 sensing structures such as IPC, carotid bodies, and central CO_2 sensitive areas are temporarily or permanently dulled by chronic prenatal exposure to hypercapnia and/or hypoxia. Adaptive, and, in some cases, irregular alterations in ventilatory reflex behavior are likely shaped by experience during the nestling period within the burrow (Boggs and Kilgore, 1983). More research on burrowing bird species at different developmental stages would increase our understanding of how natural exposure to hypercapnia and hypoxia impact the development of the HCVR and HVR. For example, it would be interesting to measure ventilatory chemoreflexes in fledgling and young adult bank swallows to determine when and how their chemosensitivity reaches the adult level. Further, it may be informative to raise an obligate burrowing species in hyperoxic or normocapnic conditions and assess whether their ventilatory reflexes remain blunted.

7. Summary

Although birds have had a long history as the subjects for studies into the details of vertebrate gas exchange, we hope that this review draws attention to the contemporary research potential of these animals to increase our understanding of respiratory neurobiology during early life. Throughout this document, our goal was to describe what is currently known for this taxa in the context of the development and maturation of breathing control and to highlight areas that are still in need of investigation. For example, despite unparalleled access to the developing embryo at any time during prenatal and paranatal life, how inspiration and expiration phases of ventilation are constructed and organized in the form of the central respiratory networks is mostly unknown. In our opinion, there is significant potential for avian models to help determine the fundamental rules of neural circuit development, both physiologically and genetically, within the respiratory-related embryonic hindbrain. Once central pattern and rhythm generation are addressed, the connections of central and peripheral chemoreceptors and their function on breathing patterns can be further elucidated with the added bonus of tackling developmental plasticity and its related clinical disorders in young animals.

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