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Buccal rhythmogenesis and CO_2 sensitivity in *Lithobates catesbeianus* tadpole brainstems across metamorphosis

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ABSTRACT

Bullfrog tadpoles ventilate both the buccal cavity and lung. In isolated brainstems, the midbrain/pons influences CO_2 responsiveness and timing of lung ventilatory bursting, depending on larval development. However, little is known about midbrain/pons influences on buccal burst patterns. As such, we investigated how removal of this region affects buccal burst shape and CO_2 responsiveness across development. We measured facial nerve activity in brainstems isolated from tadpoles during early and late developmental stages, under normal and elevated levels of CO_2. Brainstems were either left intact or transected by removing the midbrain/pons. In late stage preparations, buccal burst pattern differed between intact and reduced preparations, and bursts were responsive to elevated CO_2 in these reduced preparations. These results suggest the midbrain/pons affects tadpole buccal burst pattern and CO_2 responsiveness, perhaps similar to its influences on lung ventilation.

1. Introduction

Amphibians are unique in that ventilation of both the lungs and gills contribute to gas exchange during larval development. For both ventilatory behaviors, the motor patterns (lung and gill/buccal bursting) are generated in the medulla, and modulated by central or peripheral changes in O_2 or CO_2 (Torgerson et al., 1997; Remmers et al., 2001; Wilson et al., 2002; Gargaglioni and Milsom, 2007). The role of the midbrain/pons in modulating these patterns and in modulating ventilatory responses to CO_2 in air-breathing animals is well documented (For review see (Dutschmann and Dick, 2012)). In amphibians, the broad conclusion is that this region provides tonic excitation and shaping of lung burst patterns, and is involved in responses to changes in CO_2 (Kinkead et al., 1997; Milsom et al., 1997; Reid et al., 2000; Gargaglioni et al., 2002; Noronha-de-Souza et al., 2006; Gargaglioni et al., 2007; Santin and Hartzler, 2013). However, the role of this region in buccal burst generation and CO_2 responsiveness is unclear.

When buccal burst patterns of bullfrog tadpoles and fish have been studied in isolated preparations, results have largely supported the conclusion that, unlike lung bursts (Taylor et al., 2003a, b; Brundage and Taylor, 2010), buccal/gill bursts are not responsive to changes in CO_2 (Hedrick et al., 1991; Cote et al., 2014; Hoffman et al., 2016). Interestingly, when the midbrain/pons region is removed from the isolated preparations, buccal bursts have been reported to respond to elevated CO_2 (Torgerson et al., 1997; Remmers et al., 2001). Similarly, when isolated preparations lacking the midbrain/pons are exposed to hypoxia, buccal burst patterns change (Fournier and Kinkead, 2008). As an important caveat, reports of buccal burst changes in response to CO_2 and O_2 also indicate developmental specificity. Despite these findings, substantial questions remain regarding the emergence of buccal burst CO_2 responsiveness and if its expression depends on removal of the midbrain/pons.

Because of this, and given the prominent role of the midbrain/pons in modulating lung ventilation and chemoresponsiveness, we speculated that these regions might also function similarly to modulate buccal burst patterns. We investigated how removing this region might affect the shape and CO_2 responsiveness of buccal bursting across larval development. We hypothesized that buccal burst CO_2 responsiveness emerges as a result of removing the midbrain/pons, and that removing this region would affect buccal burst shape across development.

2. Methods

2.1. Animals and experimental preparations

All care and experimental protocols were approved by the

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Institutional Animal Care and Use Committee at the University of Alaska Fairbanks, and complied with all state and federal ethical guidelines. Tadpoles were purchased from a commercial supplier (Pond Megastore, www.pondmegastore.com) and imported under permit from the Alaska Department of Fish and Game. Animals were housed in aquaria with dechlorinated water at room temperature, and fed goldfish food daily.

Studies were performed using 22 American bullfrog tadpoles (*Lithobates catesbeianus*) separated into two groups representing different stages of larval development, as defined by external anatomy. One group represented early stages of development (early-stage), characterized by the absence of forelimbs and the presence of a tail and paddle-like hind limbs lacking joints or separated toes (n = 11). The second group represented later stages of development (late-stage), characterized by the presence of forelimbs, developed hind limbs, and partial resorption of the tail (n = 11). These groups corresponded to developmental stages VII–XII and XX–XXV, respectively, in the classification scheme of tadpole development originally proposed by Taylor and Kollros (Taylor and Kollros, 1946).

Isolated brainstem-spinal cords were prepared using our established methods (Harris et al., 2002; Taylor et al., 2003b; Brundage and Taylor, 2010). Each tadpole was anesthetized by immersion for 1–2 min in cold (4 °C) 0.2 mM tricaine methanesulfonate (MS222 in dechlorinated water buffered to pH 7.8 with $NaHCO_3$; Sigma, www.sigmaaldrich.com). The dorsal cranium and forebrain rostral to the diencephalon were resected and the fourth ventricle opened by removing the choroid plexus. The remaining brainstem and spinal cord were removed *en bloc* and further trimmed rostral to the optic tectum and caudal to the brachial nerves. During dissection, exposed tissues were superfused with cold artificial cerebral spinal fluid (aCSF) composed of (in mM) 104 NaCl, 4 KCl, 1.4 $MgCl_2$, 10 D-glucose, 25 $NaHCO_3$ and 2.4 $CaCl_2$ equilibrated with 100% O_2. The aCSF HCO_3 concentration is similar to that of plasma from tadpoles during late stages of larval development and adult frogs, but higher than that observed in plasma of aquatic tadpoles during early stages of development (Just et al., 1973). This HCO_3 concentration has been used in previous tadpole studies (Taylor et al., 2003a, b; Taylor et al., 2008) and was selected to ensure comparability with previous studies and between experimental preparations made from animals at different stages of metamorphosis.

The isolated brainstem was transferred *en bloc* to a 2.5-mL Plexiglas recording chamber and was supported, ventral side up, between coarse nylon mesh such that all surfaces were bathed with flowing, oxygenated aCSF. A reservoir of aCSF was equilibrated with $O_2 - CO_2$ mixtures that produced the desired level of dissolved gases. The fractional concentrations of O_2 and CO_2 were adjusted in the equilibration gas with a programmable gas mixer (GSM-3; http://www.cwe-inc.com), and this mixture was monitored with a CO_2 analyzer (AMETEK CD-3A; www.ametek.com). This perfusate was gravity fed from the reservoir through plastic tubing to the recording chamber allowing the isolated brainstem to be bathed with a rostral to caudal flow of gas-equilibrated aCSF at a rate of 5 mL/min. Pilot studies confirmed that gas conditions and pH of aCSF in the recording chamber matched that in the reservoir. After isolation, the brainstem was allowed to stabilize for 1 h while superfused with aCSF equilibrated with 1.5% CO_2, 98.5% O_2 (normocapnia; 23 °C; pH 7.8; P_{CO_2} ~9 mmHg; P_{O_2} ~590 mmHg). Solution hyperoxia (P_{O_2} ~590 mmHg) is likely to produce oxygen radicals, which we have shown to influence patterns of burst discharge recorded from these preparations (Harris et al., 2002). This hyperoxia, however, is consistent between treatments and is common practice with this preparation to ensure that O_2 content is sufficient to meet tissue metabolic demands.

2.2. Nerve recordings

Roots of the facial (7th cranial nerve, CN VII) or hypoglossal (2nd spinal nerve, SN II) nerves were drawn into glass suction electrodes pulled from 1-mm diameter capillary glass to tip diameters that fit the nerve roots. Whole-nerve discharge was amplified (X100 by DAM 50 amplifiers, World Precision Instruments, www.wpiinc.com; X1000 by a model 1700 amplifier, A–M Systems, www.A–Msystems.com) and filtered (60 Hz notch, 100 Hz low frequency, 1 kHz high frequency). The amplified and filtered nerve output was sent to an analog-to-digital data acquisition system (Power 1401, Cambridge Electronic Design, ced.co.uk), which sampled at 25 kHz. Data were archived in duplicate as raw neurograms of whole-nerve discharge and as an "integrated" neurogram following a digital transformation (root mean square transform at 100 ms and smoothed over 200 ms). Neurograms were acquired simultaneously from two nerves prior to transections. Such recordings were made during the initial 1 h post-isolation stabilization period and recorded continuously throughout the duration of each treatment protocol.

2.3. Transections and experimental protocol

Whole-nerve recordings were made from "intact" or "transected" brainstem preparations. In these experiments, an intact brainstem preparation consisted of a block of contiguous tissue from the optic tectum to the brachial nerve roots in the spinal cord, as prepared during initial dissection. After a 1 h recovery in normocapnia (1.5% CO_2 : 98.5% O_2, pH 7.8), brainstems were either left intact (Fig. 1a), or further transected at the level of the trigeminal nerves creating a caudal segment isolated from structures of the rostral brainstem, including the midbrain/pons. This remaining segment contained the rostral and caudal chemosensory areas (Taylor et al., 2003b) as well as the lung and buccal oscillators ((Wilson et al., 2002); Fig. 1b), but lacked the midbrain/pons region. Brainstem segments were allowed to recover for 1 h following transection before administering an experimental protocol of 15 min normocapnia, then 30 min of hypercapnia (5.0% CO_2 : 95.0% O_2, pH 7.4), followed by a recovery period of 1 h at normocapnia (Fig. 1c). Pilot studies suggested that 1 h was sufficient for recovery from transection, as burst patterns occurring after this recovery period did not change with additional exposure to baseline conditions. Nerve discharge patterns were recorded continuously before transections. Nerve recordings were paused, and nerve roots released from electrodes to facilitate transection without damaging the remaining nerves. Electrodes were reconnected during the post-transection recovery period, and recordings recommenced for the duration of the experiment.

2.4. Data and statistical analyses

For consistency, data were analyzed from 5 continuous minutes within the final portion of each gas treatment period (Fig. 1c). Pilot studies indicated that 30 min of gas treatment and 1 h of recovery after hypercapnia or transection, were sufficient to establish steady states. Like other amphibian *in vitro* preparations, intact brainstems produce distinct cranial/spinal nerve discharge patterns indicative of both buccal cavity and lung ventilation *in vivo* (Gdovin et al., 1999). Distinct patterns of burst activity within recorded neurograms were designated as either "buccal" or "lung" bursts on the basis of the amplitude of bursts within the integrated nerve activity, and the presence or absence of coincident bursting in both CN VII and SN II nerves, as previously described (Kogo et al., 1994; Gdovin et al., 1998; Reid and Milsom, 1998; Torgerson et al., 1998; Gdovin et al., 1999; Taylor et al., 2003b). Bursts with higher amplitude activity, coincident on both CN VII and SN II, were designated as "lung bursts". Bursts with lower amplitude activity in CN VII, with little or no coincident activity in SN II, were designated as "buccal bursts". Lung burst patterns and responsiveness to experimental treatments were not considered in the current analysis, and have been recently reported (Reed et al., 2018).

Buccal bursts from each treatment group were analyzed for 6 parameters. Burst average instantaneous frequency was determined from the average of all buccal cycle durations (onset of one buccal burst

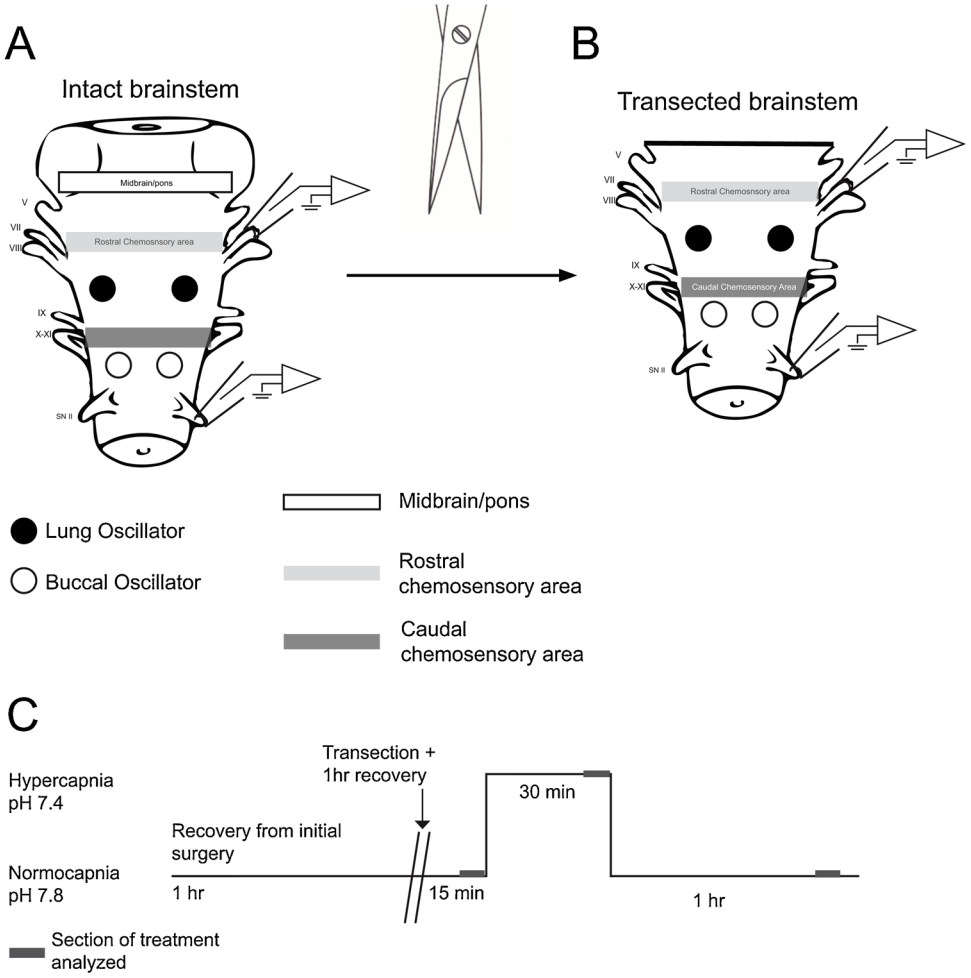

Fig. 1. Experimental preparations and protocols. (A) Intact brainstems were compared to brainstems transected (B) at the level of the trigeminal nerves (V) to remove the midbrain/pons. Burst discharges were recorded from the facial (VII) and hypoglossal (2nd spinal nerve; SN II) nerves in each brainstem preparation (intact or transected). (C) A hypercapnic gas treatment was administered followed by a normocapnia recovery treatment. The last 5 min of each gas treatment were analyzed.

to the onset of the subsequent lung or buccal burst) and is expressed as a frequency (Hz). Burst duration was measured as the time between burst onset (signal deviation from tonic background activity) and the point where the signal returned to baseline (sec). In addition, we subdivided each burst to reflect the time between burst onset and peak activity (time-to-peak, sec), and the time between peak activity and return to background (time-to-trough, sec). Burst parameters were compiled for each combination of preparation and treatment, and are reported as the mean values calculated from all bursts occurring within each observation period.

Table 1 reports all mean values calculated for early and late stage groups and each combination of gas treatment, in intact and transected preparations. Burst variables were compared using factorial two-way repeated measures analysis of variance (one-factor repetition RMANOVA; SigmaStat, www.systat.com) with post hoc Holm-Sidak pairwise multiple comparisons procedures. Overall significance level was set to $p \leq 0.05$. Values are reported as means ± standard error of the mean.

3. Results

3.1. Hypercapnia did not stimulate buccal burst frequency in intact brainstems of early or late stage tadpoles

We tested and compared the CO_2 response between intact brainstems from early (n = 6) and late (n = 7) stage tadpoles. Whole-nerve discharge was recorded from CN VII and SN II in normocapnic (1.5% CO_2; NC) and hypercapnic (5% CO_2; HC) conditions from intact preparations made from of early and late stage tadpoles. Lung and buccal bursts occurred in both early and late stage tadpoles, during both normocapnia and hypercapnia (Fig. 2a, b). Instantaneous buccal burst frequency was lower in early stage (NC = 0.65 ± 0.05 Hz) compared to late stage (NC = 1.10 ± 0.06 Hz) preparations, regardless of gas treatment (developmental stage factor $F_{1,22} = 34.93$, $p < 0.001$; Fig. 2c). Hypercapnia did not change instantaneous buccal burst frequency in early (HC = 0.66 ± 0.05 Hz) or late (HC = 1.06 ± 0.06 Hz) stage preparations (although the gas treatment factor was significant $F_{2,22} = 3.67$, $p = 0.042$; there was no between factors interaction effect $F_{2,22} = 0.622$, $p = 0.546$; and the post-hoc tests showed no significant differences: NC vs HC, p = 0.639; NR vs HC, p = 0.054; Fig. 2c). The durations of buccal bursts were longer in early (NC = 1.26 ± 0.10 s) compared to late (NC = 0.67 ± 0.03 s) stage preparations (developmental stage factor $F_{2,22} = 40.03$, $p < 0.001$), and were not affected by hypercapnia (gas treatment factor $F_{2,22} = 2.51$, $p = 0.104$; no between factors interaction effect $F_{2,22} = 0.84$, $p = 0.446$; Fig. 2d).

3.2. In transected preparations, hypercapnia stimulated the frequency of buccal burst discharge in late stage tadpoles

We transected brainstems from early (n = 5) and late (n = 4) stage

Table 1
Buccal burst characteristics. Values are listed as mean ± standard error of the mean. Symbols denote $p \leq 0.05$: a significant difference in late stage compared to early stage is indicated by (#), a difference in hypercapnia compared to baseline normocapnia (a hypercapnic response) is indicated by (†), and a difference between transection when compared to intact preparations is indicated by (*).

		Early stage			Late stage		
		Normocapnia	Hypercapnia	Recovery Normocapnia	Normocapnia	Hypercapnia	Recovery normocapnia
Instantaneous frequency (Hz)	Intact	0.65 ± 0.05	0.66 ± 0.05	0.71 ± 0.06	1.10 ± 0.06 #	1.06 ± 0.06 #	1.16 ± 0.06 #
	Transection	0.67 ± 0.07	0.82 ± 0.08	0.69 ± 0.05	0.52 ± 0.04 *	0.77 ± 0.09 † *	0.58 ± 0.06 *
Buccal burst duration (sec/burst)	Intact	1.26 ± 0.10	1.22 ± 0.09	1.17 ± 0.09	0.67 ± 0.03 #	0.70 ± 0.04 #	0.64 ± 0.03 #
	Transection	1.13 ± 0.08	0.96 ± 0.08	1.10 ± 0.93	1.58 ± 0.14 *	1.02 ± 0.19 † *	1.38 ± 0.12 *
Interburst Duration (sec)	Intact	0.31 ± 0.05	0.29 ± 0.03	0.27 ± 0.03	0.25 ± 0.02	0.27 ± 0.02	0.23 ± 0.02
	Transection	0.14 ± 0.03 *	0.09 ± 0.02 *	0.13 ± 0.03 *	0.48 ± 0.02 # *	0.41 ± 0.03 # *	0.49 ± 0.07 # *
Cycle Duration (sec)	Intact	1.59 ± 0.14	1.54 ± 0.10	1.46 ± 0.11	0.92 ± 0.04 #	0.96 ± 0.05 #	0.88 ± 0.04 #
	Transection	1.52 ± 0.14	1.25 ± 0.13	1.47 ± 0.11	1.95 ± 0.16 *	1.35 ± 0.15 † *	1.78 ± 0.16 *
Time to peak (sec)	Intact	0.59 ± 0.07	0.55 ± 0.04	0.57 ± 0.05	0.34 ± 0.02 #	0.36 ± 0.02 #	0.32 ± 0.02 #
	Transection	0.64 ± 0.11	0.52 ± 0.05	0.58 ± 0.04	0.83 ± 0.06 *	0.51 ± 0.10 † *	0.71 ± 0.06 *
Time to trough (sec)	Intact	0.67 ± 0.07	0.67 ± 0.10	0.60 ± 0.06	0.32 ± 0.01 #	0.34 ± 0.02 #	0.32 ± 0.01 #
	Transection	0.49 ± 0.06	0.45 ± 0.04	0.52 ± 0.07	0.75 ± 0.09 *	0.51 ± 0.10 † *	0.67 ± 0.06 *

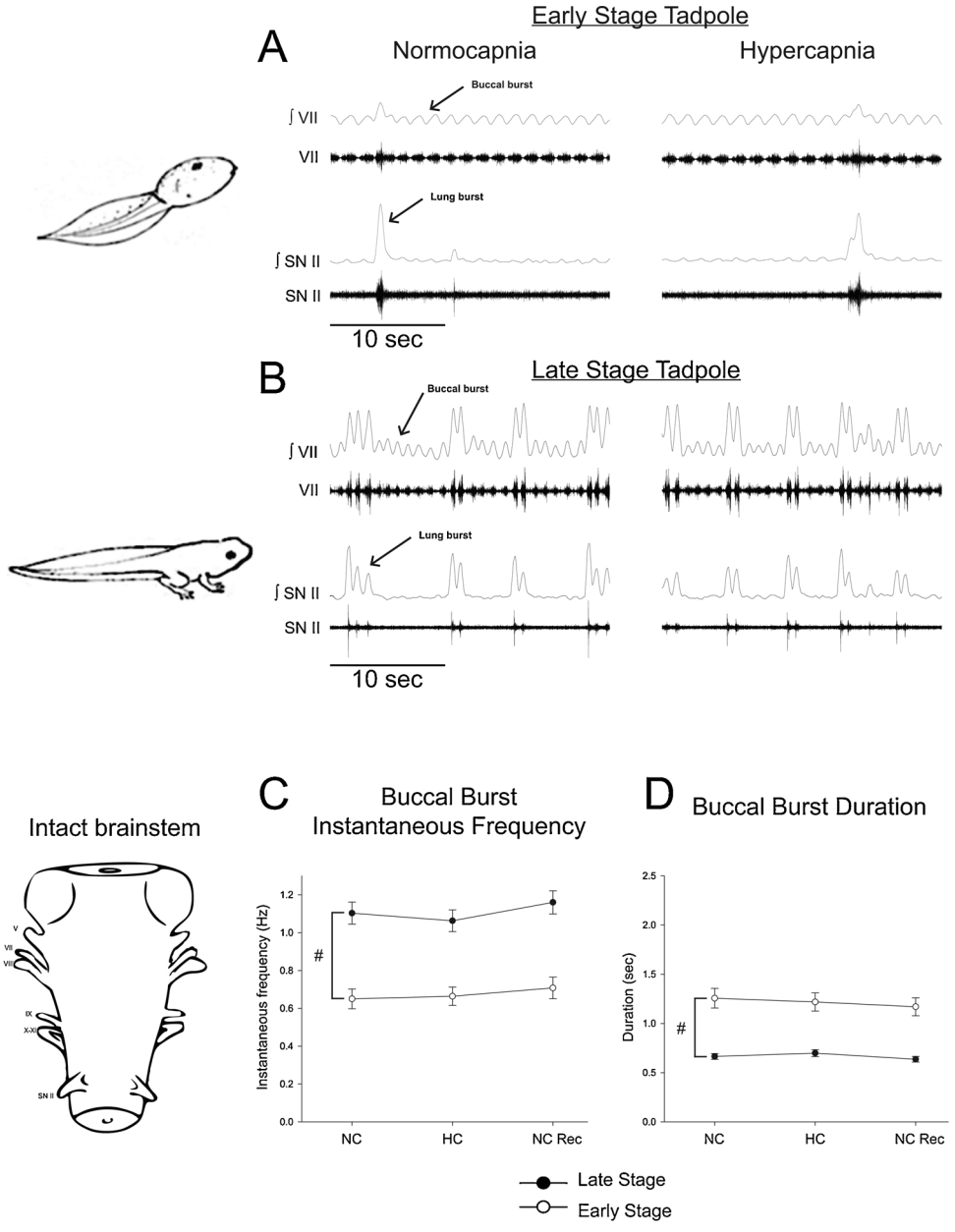

Fig. 2. In intact brainstem preparations, buccal bursts were more frequent in late than early stages, and were not responsive to hypercapnia. Whole-nerve discharge (raw and integrated "∫") was recorded from the facial (CN VII) and hypoglossal (SN II) nerves of intact brainstem preparations from early and late stage tadpoles, during normocapnic (NC), hypercapnic (HC), and normocapnic recovery (NC Rec) conditions. Both buccal and lung bursts were observed in early (A) and late (B) stage preparations during both NC and HC. (C) Buccal bursts were less frequent in preparations derived from early stage tadpoles (open circles) compared to those derived from late stage tadpoles (filled circles) regardless of CO_2 level (#). Buccal instantaneous frequency was not affected by HC in early or late stages. (D) Buccal burst duration was longer in preparations from early stage tadpoles compared to those from late stage tadpoles, regardless of CO_2 level (#).

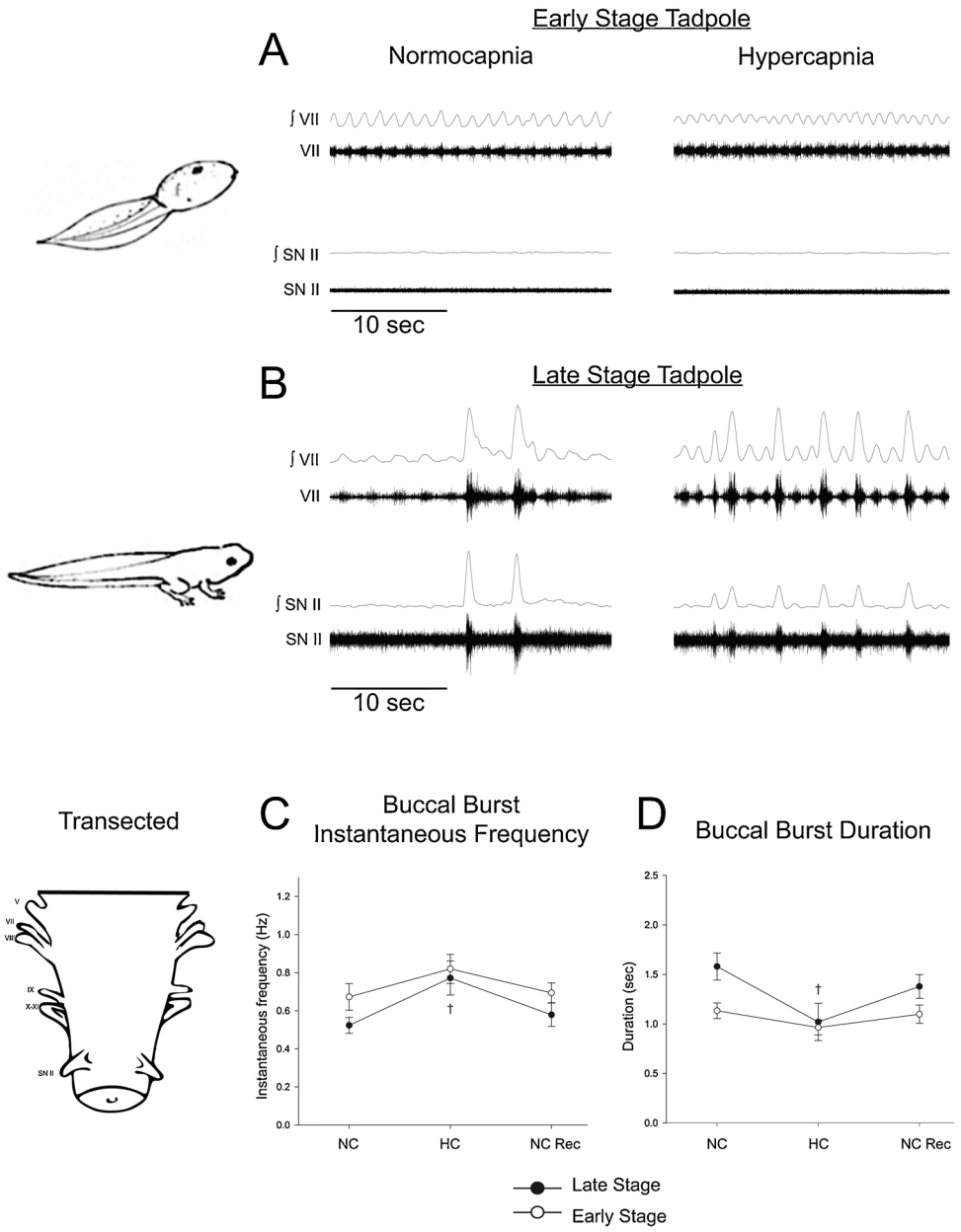

Fig. 3. Hypercapnia stimulated buccal burst frequency and decreased burst duration in transected brainstem preparations from late stage tadpoles. Whole-nerve discharge (raw and integrated "∫") was recorded from the facial (CN VII) and hypoglossal (SN II) nerves of transected preparations from early and late stage tadpoles, during normocapnic (NC) and hypercapnic (HC), and normocapnic recovery (NC Rec) conditions. Both lung and buccal bursts were exhibited by transected preparations of early (A) and late (B) stage tadpoles. (C) Buccal burst instantaneous frequency did not differ between preparations derived from early (open circles) and late (filled circles) stage tadpoles, regardless of CO_2 level. Hypercapnia modulated both buccal burst frequency (C) and duration (D) in late stage tadpoles (†).

tadpoles at the level of the trigeminal nerves, removing the midbrain/pons region. We then compared between normocapnic and hypercapnic conditions in this caudal segment. Buccal bursts were observed in transected preparations of both late and early stage groups (Fig. 3a,b). Instantaneous buccal burst frequency of transected brainstems increased with hypercapnia in late (Fig. 3c; HC = 0.77 ± 0.09 Hz; between treatments $F_{2,11} = 8.65$, $p = 0.017$) but not early stage preparations (HC = 0.82 ± 0.08 Hz; between treatments $F_{2,11} = 1.39$, $p = 0.332$). These frequency increases in response to hypercapnia did not differ between stages (between factors interaction effect $F_{2,11} = 0.532$, $p = 0.602$). Buccal burst durations did not differ between early (Fig. 3d; NC = 1.13 ± 0.08 s) and late (NC = 1.58 ± 0.14 s) stage preparations (developmental stage factor $F_{1,11} = 2.06$, $p = 0.192$) following transection. Burst durations decreased with hypercapnia in late (HC = 1.02 ± 0.19 s; between treatments $F_{2,11} = 7.8$, $p = 0.021$) but not early stage preparations (HC = 0.96 ± 0.08 s; between treatments $F_{2,11} = 2.60$, $p = 0.168$). Buccal burst duration responses to increased CO_2 did not differ between stages (between factors interaction effect $F_{2,11} = 2.22$, $p = 0.155$).

3.3. In transected but not intact preparations, hypercapnia altered buccal burst duration, but not inter-burst duration

Recordings made from transected brainstems were compared to recordings from intact early and late stage preparations with respect to instantaneous buccal burst frequency (Hz), cycle duration (sec), inter-burst duration (sec), burst duration (sec), bursts time-to-peak (sec), and burst time-to-trough (sec). Intact preparations had no instantaneous frequency response to hypercapnia, while transected preparations displayed a CO_2 response in early and late stages. In early stage preparations, instantaneous buccal burst frequency under normocapnic conditions did not differ between intact and transected preparations (Fig. 4a, transection factor $F_{1,15} = 0.22$, $p = 0.649$). Inter-burst durations differed between intact and transected preparations regardless of CO_2 (Fig. 4c, transection factor, $F_{1,15} = 15.75$, $p = 0.003$), and did not change with hypercapnia (gas treatment factor, $F_{2,15} = 1.594$, $p = 0.236$). Burst durations did not differ between intact and transected preparations (Fig. 4d, transection factor, $F_{1,15} = 0.60$, $p = 0.459$). Hypercapnia did not significantly reduce buccal burst duration in

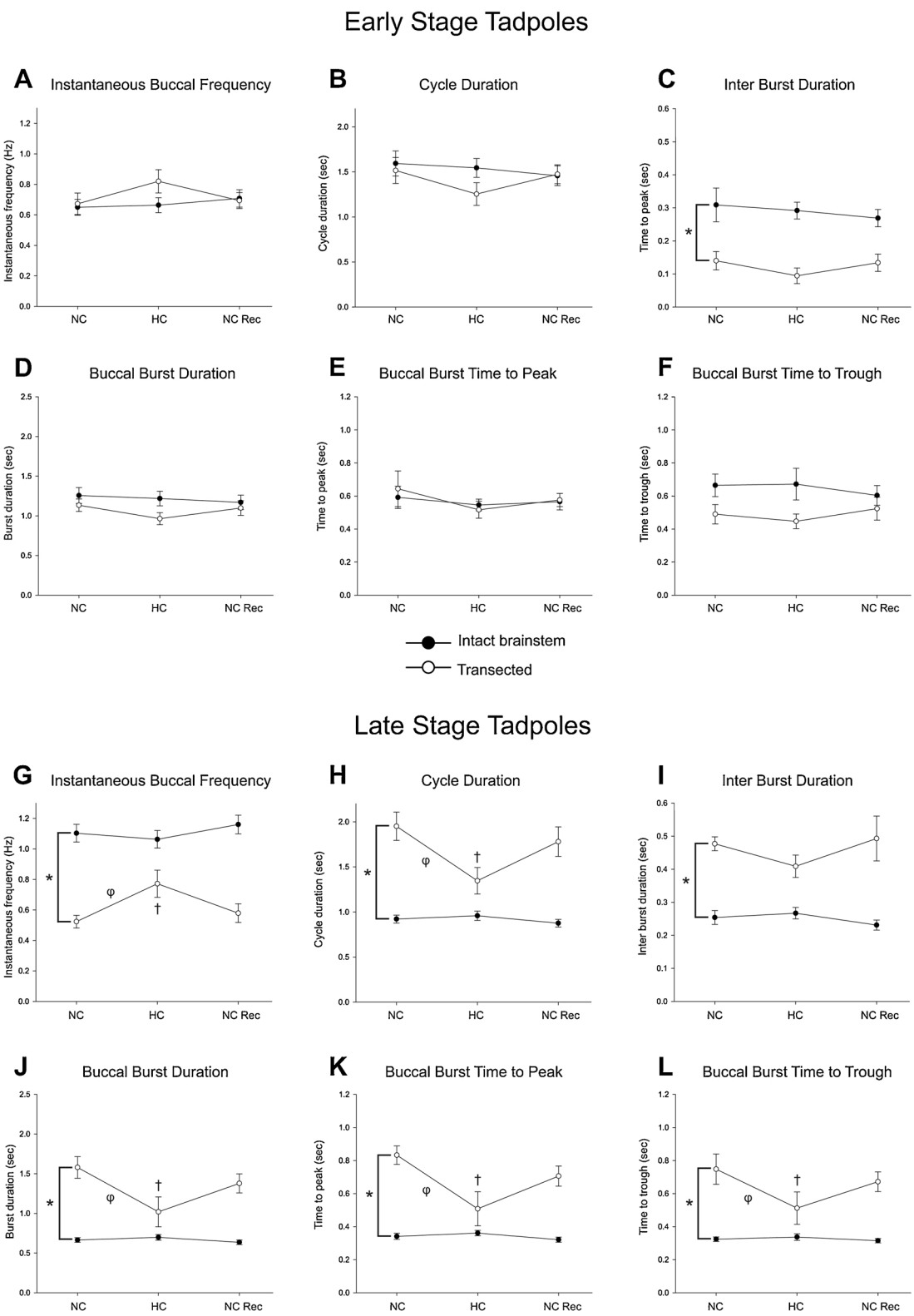

Fig. 4. Comparison of buccal burst parameters from intact and transected brainstems derived from early and late stage tadpoles. Brainstem preparations (intact = filled circles; transected = open circles) were compared with respect to: burst frequency; cycle duration; inter-burst duration; burst duration; time-to-peak; and time-to-trough. Panels A–F refer to preparations from early stage tadpoles. Hypercapnic responses are indicated with (†), and differences between intact and transected brainstem preparations are indicated by (*). Panels G–L refer to preparations from late stage tadpoles. Hypercapnic responses are indicated with (†), differences between intact and transected brainstem preparations are indicated by (*), and significant between factors interaction effects (difference in hypercapnic responses) are indicated by (φ).

transected preparations (gas treatment factor $F_{2,15} = 3.46$, $p = 0.058$). In early stages, the time-to-peak did not differ between intact and transected preparations (Fig. 4e; NC = 0.59 ± 0.07 and 0.64 ± 0.11 s, respectively; transection factor $F_{1,15} = 0.15$, $p = 0.710$), nor did the time-to-trough (Fig. 4f; HC values intact = 0.67 ± 0.07 s and transected = 0.49 ± 0.06 s; transection factor $F_{1,15} = 1.76$, $p = 0.215$).

In late stages, instantaneous buccal burst frequency was lower in transected compared to intact preparations (Fig. 4g, transection factor, $F_{1,18} = 34.82$, $p < 0.001$). Transected preparations displayed a hypercapnic response, but the intact preparations did not, which was reflected by a significant interaction effect (between factors $F_{2,18} = 9.48$, $p = 0.002$). Similar to early stages, inter-burst duration during normocapnia differed between intact and transected late stage preparations (Fig. 4i, transection factor, $F_{1,18} = 43.08$, $p < 0.001$), but neither displayed a hypercapnic response (transection factor, $F_{2,18} = 0.88$, $p = 0.433$). Hypercapnia reduced burst duration in transected but not intact preparations, which was confirmed by a significant interaction effect (Fig. 4j; between factors $F_{2,18} = 15.72$, $p < 0.001$). Buccal burst duration was higher in transected compared to intact preparations (transection factor, $F_{1,18} = 44.74$, $p < 0.001$). This larger duration was due to increases in both the time-to-peak (NC values intact = 0.34 ± 0.02 s and transection = 0.83 ± 0.06 s) and time-to-trough (NC values intact = 0.32 ± 0.01 s and rostral = 0.87 ± 0.06 s) when compared to intact preparations (Fig. 4k and 4 l; time to peak transection factor $F_{1,18} = 58.06$, $p < 0.001$; time to trough transection factor $F_{1,18} = 29.92$, $p < 0.001$). The decrease in buccal burst duration during hypercapnia in transected preparations was also due decreases in both time-to-peak and time-to-trough (Fig. 4k and 4 l; HC = 0.51 ± 0.10 s and HC = 0.51 ± 0.10 s respectively; time-to-peak between factors $F_{2,18} = 13.36$, $p < 0.001$; time-to-trough between factors $F_{2,18} = 13.30$, $p < 0.001$).

4. Discussion

4.1. Buccal burst chemoresponsiveness and its physiological relevance

Together, previous studies suggest several things about central CO_2 sensitivity as a property of the amphibian medulla. Such sensitivity seems to be present across stages of larval development rather than being a strictly emergent property of metamorphosis. Larger circuit influences between brain regions may mask this inherent sensitivity, potentially influenced by development. We have previously demonstrated that the isolated bullfrog medulla is sensitive to CO_2, but that the lung burst response to CO_2 during early larval development is likely masked by input from the caudal medulla (Reed et al., 2018). Therefore, in the current study, we considered that brainstem network influences might also underlie discrepant findings regarding buccal responsiveness to hypercapnia.

The current results support the conclusion that buccal bursting in bullfrog tadpoles can be responsive to CO_2, but that this responsiveness depends on the experimental preparation. When the midbrain/pons were removed, a buccal response to hypercapnia occurred that was not present in the larger "intact" brainstem preparation. Additionally, differences in the duration and pattern of buccal bursting were apparent when late stage transected preparations were compared to intact preparations (Fig. 4G–L).

The generation of both buccal and lung rhythms has been attributed to distinct neural oscillators (Wilson et al., 2002). Within the central nervous system, the midbrain/pons and the lung oscillator sense and respond, respectively, to changes in CO_2 (Kinkead et al., 1997; Torgerson et al., 1997, 2001; Taylor et al., 2003b; Noronha-de-Souza et al., 2006; Biancardi et al., 2008; Santin and Hartzler, 2013), and both of these areas are likely synaptically connected to the buccal oscillator. Coupling between buccal and lung oscillators has been suggested by studies *in vitro* (Wilson et al., 2002; Vasilakos et al., 2005, 2006) and in computational models (Bose et al., 2005; Horcholle-Bossavit and Quenet, 2009). Given that the lung oscillator is likely glutamatergic (Chen and Hedrick, 2008), the buccal oscillator probably receives some excitatory drive from the lung oscillator, which is also consistent with computational models (Bose et al., 2005; Horcholle-Bossavit and Quenet, 2009). Removal of the midbrain/pons increases buccal burst duration (Fig. 4j), suggesting inhibitory connections to the buccal oscillator, in addition to the aforementioned tonic excitatory connections. Therefore, the responsiveness of buccal bursting following the removal of the midbrain/pons may be due to influences of excitatory connections from the CO_2-responsive lung oscillator. As such, inhibition of the buccal oscillator provided by the midbrain/pons would mask or counteract the excitation from the lung oscillator during hypercapnia. This is one possible explanation for the collective results demonstrating that buccal rhythms are not responsive to CO_2 in intact isolated brainstems, but are responsive to CO_2 when the midbrain/pons has been removed (Torgerson et al., 1997; Remmers et al., 2001). While this is the case in bullfrog tadpoles, in intact isolated brainstems of *Rana esculenta* (European Edible Frog), removal of the midbrain/pons is not necessary for some buccal CO_2 response, as hypercapnia lengthens inter burst duration (Quenet et al., 2014).

It is important to note that this is only one possible explanation for conflicting reports of the presence or absence of buccal responses to hypercapnia in isolated brainstem preparations, as the buccal oscillator itself may be sensitive to CO_2. There is evidence for chemosensitive neurons located near the buccal oscillator, however, destruction of these neurons did not appear to affect buccal rhythms or buccal CO_2 responses (Taylor et al., 2003a). Given the lack of evidence for local buccal chemosensitivity, and the fact that responsiveness is not observed unless the midbrain/pons is removed, we think it is more likely that buccal CO_2 responsiveness is mainly a consequence of lung oscillator influences rather than a result of intrinsic chemoreceptive capability of the buccal oscillator.

We have recently demonstrated that output of the lung oscillator of early stage tadpoles becomes more noticeably CO_2 responsive after a transection manipulation (Reed et al., 2018). Similarly, although lung ventilation is rare/absent during early stages of larval development, the capacity to generate lung ventilation *per se* is present, and is revealed as precocious burst activity with disinhibition (Straus et al., 2000). Thus, the tadpole respiratory system has the capacity to respond to CO_2 across development (Taylor et al., 2003b; Brundage and Taylor, 2010; Taylor and Brundage, 2013; Reed et al., 2018), but expression of this chemosensitive capacity as responsiveness to CO_2 in the air-breathing motor pattern is limited by inputs from the caudal medulla. This is revealed when transection removes these inputs (Reed et al., 2018). The contribution of air-breathing to CO_2 excretion in tadpoles is low, with lungs of air-breathing tadpoles excreting less than 2% of total metabolic CO_2 production, rising to a maximum of only 20% in adult terrestrial bullfrogs (Burggren and West, 1982). In contrast, lung inflation is actively regulated and has a profound influence on buoyancy, locomotion, feeding and vulnerability to predation in aquatic tadpoles (Gee and Waldick, 1995; Ultsch et al., 1999; Gee and Rondeau, 2012). We speculate that limiting lung ventilation and responsiveness to CO_2 is adaptive during early stages of larval development, such that this capacity is normally suppressed and is only apparent when suppression is removed.

In the present study, we again illustrate a CO_2 responsive capacity of a respiratory motor pattern that is normally suppressed, but revealed by a transection manipulation. Unlike the limited contributions made by lung ventilation, cutaneous exchange is the primary route of CO_2 excretion in amphibians. Exchange across the gill accounts for as much as 40% of the total of CO_2 excretion in aquatic tadpoles (Burggren and West, 1982). Convection of water across the gill is produced by the buccal force pump and it may seem counterintuitive, given the importance of gill CO_2 exchange, that buccal burst frequencies are unresponsive to CO_2. However, as with our consideration of lung ventilation, suppressed responsiveness of buccal burst generation to CO_2

may be adaptive if the net costs of increasing gill ventilation outweigh the benefits of increasing CO_2 excretion.

Given the high density and viscosity of water, the energetic costs of gill ventilation and the incremental costs to increase water convection are high (Farrell and Steffensen, 1987). In excess of 40% of O_2 uptake occurs across the gills of aquatic tadpoles (Burggren and West, 1982). Given the relatively low solubility of O_2 in water, and resulting low O_2 content of water, it is generally accepted that water convection across the gill is modulated and matched to demands for O_2 extraction, and exceeds that required for CO_2 excretion. Furthermore, metabolically produced CO_2 is primarily transported as plasma bicarbonate, and CO_2 exchange at the gill requires catalyzed dehydration of plasma bicarbonate within erythrocytes to form physically dissolved CO_2 and the subsequent diffusion of CO_2 across the gill epithelium. It is likely that bicarbonate entry into the erythrocyte in exchange for intracellular chloride, rather than branchial CO_2 diffusion or blood/water convection, is the rate-limiting process in gill CO_2 excretion in amphibians, as it is in fish (Perry, 1986). If gill ventilation is not limiting gill CO_2 excretion, it would seem adaptive to suppress energetically demanding buccal burst frequency responses which carry a high metabolic burden but limited impact on overall CO_2 exchange.

The current results demonstrate a complimentary finding to that we have shown in regard to lung ventilatory burst discharge (Reed et al., 2018). Lung burst responsiveness to CO_2 occurs, and clearly serves metabolic needs for gas exchange in later developmental stages. On a system level, responsiveness is constrained in early developmental stages when such responsiveness contributes little to metabolic needs and may have substantial negative consequences. We show that buccal rhythm may have the capacity to respond to CO_2, but this capacity is suppressed in the intact network, and revealed only after a transection manipulation. It is not known if this represents direct activation of the buccal oscillator by CO_2 sensors, or activation of the lung oscillator and subsequent activation of the buccal oscillator by the lung oscillator (Wilson et al., 2002; Bose et al., 2005; Vasilakos et al., 2006; Baghdadwala et al., 2015). Again, on a system level, responsiveness is constrained, but now across all stages of tadpole development. We again speculate that such responsiveness contributes little to excretion of metabolically produced CO_2 across all stages of development in aquatic tadpoles, and that responsiveness may have substantial negative consequences by enhancing metabolic costs of water convection. We presume that, as it exists, buccal rhythm responsiveness to CO_2 has an adaptive benefit. We expect that this benefit may be revealed under circumstances or developmental stages other than those tested in the current study.

4.2. The midbrain/pons affects buccal burst shape and frequency in late stage tadpoles

Several studies have explored the role of the midbrain/pons in the control of lung ventilation in amphibians, and have supported a broad conclusion that it provides tonic excitation and burst shaping to lung rhythmogenesis (Kinkead et al., 1997; Milsom et al., 1997; Reid et al., 2000; Gargaglioni and Milsom, 2007). However, the midbrain/pons effects on buccal burst pattern are not well characterized. In transected early staged preparations, only inter-burst duration differed from intact preparations (Fig. 4A–F). In transected late staged preparations, all measured buccal burst parameters differed from intact preparations, including instantaneous burst frequency, burst duration, and inter-burst duration (Fig. 4G–L). The increased buccal burst duration observed in late stage preparations was due to an increase in both time-to-peak and time-to-trough (Fig. 4j-l), which is similar to the effect transection has on lung burst responses (Reed et al., 2018). The effect of removing the midbrain/pons on both buccal and lung bursting seems similar. Thus, it is tempting to suggest that the midbrain/pons plays a similar role in both lung and buccal burst patterns, such as providing tonic excitation and burst shaping.

The fact that we observed no differences in frequency or burst shape (duration, time-to-peak, time-to-trough) between transected and intact preparations of early stage animals suggests that the midbrain/pons may not have a large influence on buccal bursting during early stages of larval development. Of note, the observed influence of the midbrain/pons on buccal bursting occurs in preparations from larval stages in which gills have been reabsorbed. It is possible that this is an emerging role in late stage animals that may affect the frequency, timing, or even episodic nature of lung bursts. In late stage or juvenile tadpoles, either removal or pharmacological blockade of the portion of brainstem containing the buccal oscillator disrupts lung burst episodes, reduces the frequency of lung events, and increases lung burst duration (Kottick et al., 2013; Baghdadwala et al., 2015; Reed et al., 2018). Bathing isolated tadpole brainstems in chloride-free solutions to abolish buccal bursting also decreases the occurrence of lung bursts, disrupts lung episodes, and increases lung burst duration (Galante et al., 1996; Broch et al., 2002; Vasilakos et al., 2006; Leclere et al., 2012). While further work is warranted, we propose that an increasing influence of the midbrain/pons on buccal bursting throughout development serves largely to coordinate timing and shape of lung ventilation following reabsorption of the gills in late stage tadpoles. This supports the conclusion that the midbrain/pons influences neurons of the caudal medulla for timing and creation of lung episodes (Kinkead et al., 1997).

4.3. Buccal rhythms are responsive to CO_2, but only in the absence of the midbrain/pons

We conclude that conflicting reports of the absence or presence of buccal CO_2 responsiveness are largely due to methodological differences (Torgerson et al., 1997; Remmers et al., 2001; Taylor et al., 2003b). Current results are consistent with our previous findings that hypercapnia does not affect buccal frequency in intact preparations of either early or late staged tadpoles, as these preparations were tested while the midbrain/pons was intact (Taylor et al., 2003b). Also consistent with previous observations derived from similarly reduced preparations, we observe that buccal bursting in transected preparations is responsive to CO_2 (Torgerson et al., 1997; Remmers et al., 2001). We observe this CO_2 response in late staged preparations, whereas previous studies report this effect only in earlier developmental stages (Torgerson et al., 1997; Remmers et al., 2001). Despite these minor discrepancies, we propose that the current results provide resolution to the conflicting accounts of buccal responsiveness in tadpole brainstem preparations. Collectively, results support the conclusion that buccal rhythms produced in isolated brainstem preparations of larval tadpoles can be responsive to hypercapnia, but this responsiveness depends on characteristics of the experimental preparation.

5. Conclusion

Buccal rhythms produced by brainstems isolated from early and late stage tadpoles were unresponsive to hypercapnia. However, in brainstems preparations derived specifically from late stage tadpoles, and which lacked the midbrain/pons, the frequency of buccal bursts increased in hypercapnic conditions. We conclude that during all stages of larval development, bullfrog tadpole brainstems possess sensitivity to CO_2 that can influence buccal burst activity, but that CO_2 responsiveness is obscured by modulation contributed from structures in the midbrain/pons. The current results help clarify previous contradictory findings, and provide insight into an important role of midbrain/pons in buccal burst pattern formation.

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