RESEARCH ARTICLE

Reversible brain inactivation induces discontinuous gas exchange in cockroaches

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SUMMARY

Many insects at rest breathe discontinuously, alternating between brief bouts of gas exchange and extended periods of breath-holding. The association between discontinuous gas exchange cycles (DGCs) and inactivity has long been recognised, leading to speculation that DGCs lie at one end of a continuum of gas exchange patterns, from continuous to discontinuous, linked to metabolic rate (MR). However, the neural hypothesis posits that it is the downregulation of brain activity and a change in the neural control of gas exchange, rather than low MR *per se*, which is responsible for the emergence of DGCs during inactivity. To test this, *Nauphoeta cinerea* cockroaches had their brains inactivated by applying a Peltier-chilled cold probe to the head. Once brain temperature fell to 8°C, cockroaches switched from a continuous to a discontinuous breathing pattern. Re-warming the brain abolished the DGC and re-established a continuous breathing pattern. Chilling the brain did not significantly reduce the cockroaches' MR and there was no association between the gas exchange pattern displayed by the insect and its MR. This demonstrates that DGCs can arise due to a decrease in brain activity and a change in the underlying regulation of gas exchange, and are not necessarily a simple consequence of low respiratory demand.

INTRODUCTION

Insects display a wide range of gas exchange patterns, from continuous gas exchange, where O_2 and CO_2 can move freely between the insect's air-filled tracheal system and the atmosphere, to profoundly intermittent patterns as epitomised by the discontinuous gas exchange cycle (DGC). The DGC is a gas exchange pattern that is characterised by three phases: the closed (C) phase, when insects close their spiracles and gas exchange is restricted; the flutter (F) phase, when the spiracles open and close briefly allowing for O_2 uptake and limited CO_2 release; and an open phase when O_2 and CO_2 are exchanged freely between the insect and atmosphere. The long breath-hold periods typical of the DGC have led many researchers to speculate about the possible adaptive benefits of this gas exchange pattern, as well as the mechanism underlying its emergence. The protracted closure of spiracles during the DGC's C phase and intermittent opening during the F phase will reduce an insect's rate of respiratory water loss (e.g. Matthews and White, 2012; Williams et al., 2010), and there is evidence that this confers increased fitness during periods of starvation and water restriction, at least in cockroaches *Nauphoeta cinerea* (Schimpf et al., 2012). Similarly, low tracheal O_2 levels during the C and F phases of the DGC have been observed in several species (Hetz and Bradley, 2005), and have been suggested to ameliorate toxic levels of oxygen free radicals when the insect's MR is low (Bradley, 2000). However, this hypothesis may be untenable in light of direct measurements of reactive oxygen species (ROS) production in moth pupae displaying DGCs, showing that ROS production actually decreases with increasing tracheal O_2 levels (Boardman et al., 2011). Two non-adaptive hypotheses, the emergent property hypothesis (Chown and Holter, 2000) and the neural hypothesis (Matthews

and White, 2011a), have since been put forward to explain the origins and distribution of DGCs within the Insecta based on the observation that DGCs coincide with periods of inactivity. These hypotheses assume that DGCs occur due to the interaction between two respiratory systems attempting to independently regulate internal CO_2 and O_2 levels when respiratory demand is low (emergent property hypothesis), or when respiratory control falls to ganglia in the thorax and abdomen following the downregulation of brain (cephalic ganglion) activity (neural hypothesis).

Periodic breathing can only occur if the animal's MR is low enough that internal O_2 reserves and CO_2 buffering capacity can support respiration for the duration of an extended breath-hold period. This suggests that DGCs should be restricted to periods of low MR. Because insects known to display DGCs adopt a continuous pattern of gas exchange when active and tend to adopt DGCs when they are inactive, it is plausible that the DGC is simply part of a continuum of gas exchange patterns regulated by respiratory demand and MR (Bradley, 2007). However, the coincidence between inactivity, low metabolic rate and DGCs does not mean that it is low MR *per se* that drives the emergence of this pattern. Indeed, there is considerable overlap between the MRs of insects displaying DGCs and continuous gas exchange patterns (Matthews and White, 2012; Schimpf et al., 2012; Williams et al., 2010). While it is perhaps unsurprising that the respiratory requirements of a resting insect can be satisfied equally well by either a continuous or a discontinuous gas exchange pattern, it does raise the question: why change to a discontinuous pattern of gas exchange during periods of inactivity instead of regulating a low continuous rate of gas exchange? Is the DGC simply the default pattern of gas exchange adopted by an insect when their MR is low, or is the DGC the result of an underlying change in

how gas exchange is regulated? To disentangle the effect of the insect's MR and neural inactivity on gas exchange pattern, we used a cold probe to regulate the activity of a cockroach's brain independently of its body temperature and, therefore, MR.

MATERIALS AND METHODS
Animals

Speckled feeder roaches *Nauphoeta cinerea* (Olivier 1789) were purchased from Live Foods Unlimited (Gold Coast, QLD, Australia) and kept in plastic terraria maintained at room temperature (25°C) under a natural light regime. Food (carrot) was provided *ad libitum*. All cockroaches were weighed to 0.1 mg immediately before measurement.

Brain temperature regulation

Brain temperature was manipulated using a custom-built thermoelectric cooler (Fig. 1). A 40×40 mm Peltier element (TE-127-1.4-1.5, TE Technology, Traverse City, MI, USA) driven by a TC-24-12 temperature controller (TE Technology) was used to chill a 12.7×50×70 mm copper block. The hot side of the Peltier element was bolted to a second copper block mounted on a 300×450×12.7 mm M6 threaded aluminium breadboard (MB3045/M, Thorlabs, NJ, USA), which acted as a heat sink. Two 80×80 mm computer fans mounted on the breadboard ensured rapid heat dissipation. The entire copper block assembly was then encased in moulded insulation made from GP330 rigid polyurethane foam (Dalchem, VIC, Australia). The cold probe itself was made from a 5 mm diameter silver rod with one end cut on a slight angle. It was fixed vertically within a socket in the centre of the chilled copper block. A miniature NTC thermistor (0.97 mm diameter, QTMB-16B3, Quality Thermistor, Boise, ID, USA) was then inserted into a hole drilled in the side of the silver rod, near the tip. The hole was backfilled with thermal paste (TP-1, TE Technology). The thermistor was connected to the control sensor input of the temperature controller, allowing the temperature at the tip of the cold probe to be regulated accurately. A raised Perspex platform was built over the copper block assembly with a hole in its centre through which the cold probe projected.

A cockroach was secured within the respirometry chamber as follows. First, the cockroach was cold knocked-out by placing it in a freezer for 7 min. It was then affixed by its dorsal surface to a small Perspex tile using a polyvinylsiloxane casting material (President light body dental impression material, Coltène Whaledent,
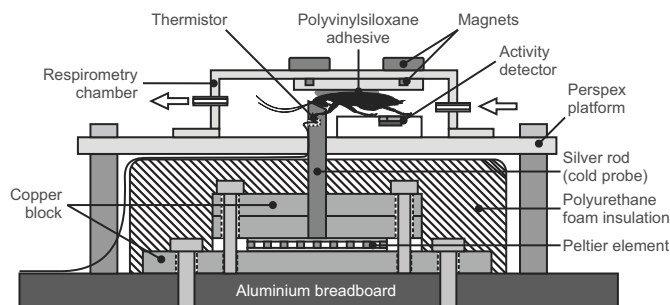

Fig. 1. Schematic of the apparatus used to control the temperature of the cockroach's brain. A silver rod held in contact with the cockroach's head was cooled by a Peltier element located between two copper blocks. The circuit driving the Peltier element regulated the temperature of a bead thermistor inserted near the tip of the rod. Arrows indicate direction of airflow through the respirometry chamber.

Altstätten, Switzerland). Two 3 mm diameter rare-earth magnets embedded in the tile allowed the cockroach to be positioned and moved within the respirometry chamber using two magnets placed on the outside of the chamber. The respirometry chamber consisted of a 70×30×15 mm rectangular transparent polystyrene enclosure that was open on the bottom. Once the cockroach was secured to the roof of the chamber using the magnets, the chamber was sealed onto the Perspex platform using a ring of Blu-Tack® modelling putty (Bostik, Thomastown, VIC, Australia). The cockroach was held over a polystyrene foam block and then manoeuvred by manipulating the magnets outside the chamber until the front of its head was in contact with the angled tip of the cold probe. A small amount of thermal paste applied to the end of the probe ensured good thermal contact between the cockroach's head and the cold probe. All experiments were conducted with the apparatus at room temperature (25°C). The experiment began with a settling time of 30 min, with the temperature of the cold probe set at 25°C. Following this, the probe's temperature was decreased to −0.6°C for 2 h, before being stepped through 2, 4, 6, 10, 15, 20 and 25°C, with 30 min given at each temperature.

To determine whether chilling the head had an effect on whole body temperature, an infrared (IR) camera (Avio TVS-500, Nippon Avionics, Tokyo, Japan) was used to image the entire cockroach through a thin polyethylene window in the side of the respirometry chamber. Images were recorded at each cold probe temperature used during the experiments. Following respirometry, brain temperature was measured directly by inserting a 25 gauge needle containing a calibrated T-type thermocouple through the top of the respirometry chamber and in through the top of the cockroach's head while it remained in contact with the cold probe. The temperature of the probe was then changed while head temperature was measured using a digital thermometer (Model 52, Fluke Corp., Everett, WA, USA).

MR and gas exchange pattern measurement

Dry, CO_2-free air was produced by drawing outside air through Drierite (W. A. Hammond Drierite Co., Xenia, OH, USA), soda lime, Drierite columns. It was then passed through a GFC171S 0–1000 ml min^{-1} mass flow controller (Aalborg, Orangeburg, NY, USA) and into the respirometry chamber at 250 ml min^{-1}. The excurrent air from the chamber was then passed through a LI-820 CO_2 analyser containing a 5 cm optical path (LI-COR, Lincoln, NB, USA). A SFH 9202 reflective interrupter (Osram Opto Semiconductors, Regensburg, Germany) placed in the foam block underneath the cockroach was used to detect abdominal pumping movements associated with ventilation. The SFH 9202 was connected to a custom-built circuit that produced a voltage change proportional to the abdomen's distance from the sensor. Voltage outputs from the CO_2 analyser (p.p.m.), mass flow controller (ml min^{-1}), abdominal activity detector (mV) and temperature controller (set and control temperatures; °C) were sampled at 10 Hz by an 8/30 Powerlab (ADInstruments, Bella Vista, NSW, Australia) and recorded by a laptop running Chart ver. 7 software (ADInstruments).

Statistical analysis

Results are presented as means ± 95% CI. Statistical analysis was conducted using StatistiXL ver. 1.8 (www.statistixl.com).

RESULTS

Simultaneous measurements of gas exchange pattern and rate of CO_2 production ($\dot{V}_{CO_2}$, a proxy for MR) were made on 10
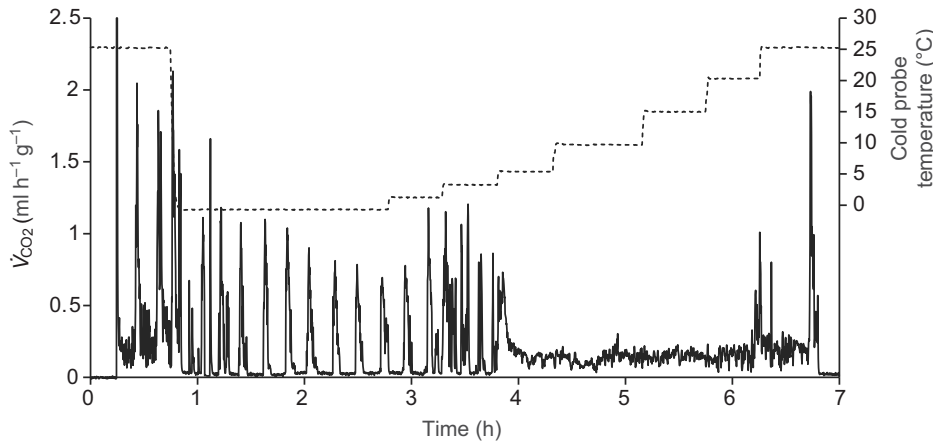
Fig. 2. Example trace recorded from a 537 mg female cockroach showing gas exchange pattern (solid line) in relation to the temperature of the cold probe in contact with the cockroach's head (dashed line). Initially, the cockroach's rate of CO_2 release ($\dot{V}_{CO_2}$) is high and continuous during bouts of vigorous activity. Gas exchange becomes discontinuous once the cockroach's head is cooled, returning to continuous as it is rewarmed. Spikes in $\dot{V}_{CO_2}$ in the first and last hours of the experiment are due to bouts of vigorous activity.

cockroaches (five male, five female) with a mean body mass of 547±29 mg. Initially, all individuals displayed vigorous activity when secured within the respirometry chamber and gas exchange was continuous. The cold probe was then chilled to −0.6°C, stabilising at this temperature within 5.5 min. All cockroaches rapidly became inactive and began to display DGCs within ~15 min (Fig. 2). After 2 h at −0.6°C, the temperature of the cold probe was increased every 30 min to 2, 4 and then 6°C. During this period the discontinuous gas exchange pattern became erratic, and the cockroaches showed signs of activity. Once head temperature had been rewarmed to 6°C, gas exchange had become continuous in all individuals (Fig. 2). Once the temperature of the cold probe had been increased to 20–25°C, the cockroaches would once again display bouts of activity. Cockroaches did not appear to be permanently affected by this treatment, showing normal activity at the end of the experiment. No significant difference was detected between the MRs of cockroaches displaying DGCs or continuous gas exchange patterns (Fig. 3). The only significant difference found between MRs at different temperatures was between 10 and 25°C (full factorial ANOVA, $P=0.046$) when all individuals were breathing continuously.

Head temperature

IR thermography revealed that chilling the head had little effect on the cockroach's overall body temperature (Fig. 4). The temperature of the abdomen was unchanged by setting the cold probe to −0.6°C, remaining at 25°C. *In situ* cephalic ganglion temperature was measured from four cockroaches. The relationship between cold probe temperature and ganglion temperature was $T_{brain}=0.63\times T_{probe}+8.47$ ($R^2=0.99$). Thus, when the cold probe was

set to −0.6°C, the temperature of a cockroach's cephalic ganglion was ~8°C. Previous work on using cold blocks on the cockroach nervous system has shown that a temperature of 7°C is sufficient to block nerve conduction in the cockroach *Periplaneta americana* (Farley et al., 1967).

Fig. 4. Infrared thermography images of a cockroach with its head in contact with the cold probe (far left) at −0.6, 6, 15 and 25°C. The schematic (top) illustrates the cockroach's position within the chamber.
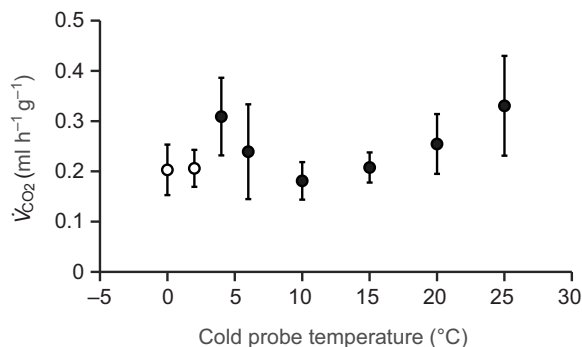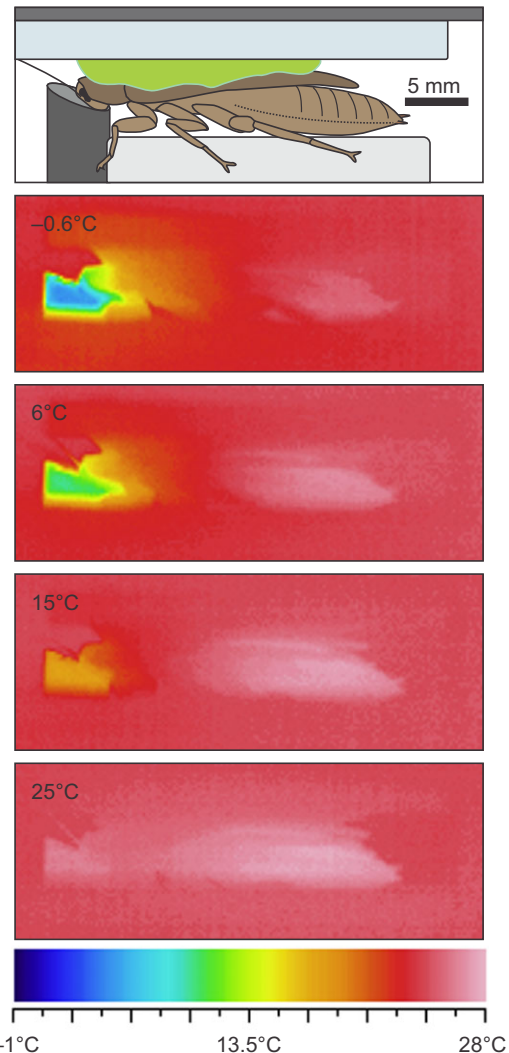

Fig. 3. Mean (±95% CI) metabolic rate of cockroaches displaying discontinuous (white circles) and continuous gas exchange (black circles).

DISCUSSION

The results presented here demonstrate that DGCs can be induced in a cockroach by reversibly inactivating its brain with a cold probe. The absence of a correlation between MR and the breathing pattern adopted demonstrates that DGCs can arise as a result of a change in the underlying neurological control of gas exchange, rather than as a direct consequence of low respiratory demand driving how gas exchange is regulated. While MR must influence the frequency of any gas exchange pattern through its effect on tracheal partial pressures of O_2 and CO_2 (Förster and Hetz, 2010; Matthews and White, 2011b; Miller, 1960), the present study shows that MR is not necessarily the critical factor determining whether an insect breathes continuously or discontinuously. This finding is in agreement with research on a range of species that has found no correlation between an insect's MR and their gas exchange pattern (Marais and Chown, 2003; Matthews and White, 2012; Williams et al., 2010).

The ability to induce DGCs reversibly in cockroaches by selectively cooling their brain provides tentative support for the neural hypothesis: that DGCs are associated with a sleep-like state in insects, involving the downregulation of activity in their cephalic ganglia (Matthews and White, 2011a). In the present study, an active brain was associated with intermittent spontaneous movement and a continuous pattern of abdominal pumping and gas exchange (Fig. 2), suggesting that when the cockroach is alert, the cephalic ganglion stimulates or coordinates the thoracic and abdominal ganglia to drive a continuous breathing pattern, but during brain inactivation this stimulation is absent, resulting in a discontinuous pattern of gas exchange. While DGCs coincide with natural brain inactivity in diapausing moth pupae (Hetz, 2007; Van Der Kloot, 1955), it remains to be shown that this same mechanism is responsible for the emergence of DGCs in other insects under normal circumstances. However, if the neural hypothesis is correct, it may explain why intact insects can show a high degree of variability in their DGCs over time, while insects that are decapitated, deeply cooled or in diapause appear to display DGCs that are more regular in frequency. Intact insects can significantly change their DGCs over several hours (burst frequency and peak CO_2 release rate per burst) when both the insect's MR and environmental conditions remain constant (Chown, 2001). These changes could be associated with gradual decline in arousal due to the insect settling into a quiescent state over time. Intact insects are also susceptible to environmental disturbances (noise, vibration, etc.) that can cause them to alter their gas exchange behaviour (Hadley and Quinlan, 1993; Kestler, 1985), potentially accounting for some of the variability in their gas exchange patterns. In comparison, the DGCs displayed by decapitated insects that have been rendered insensible to such disturbances are far more consistent. For example, the standard deviation of DGC frequency in three ant species is 1.7 to 3.8 times lower when they are headless compared with when they are intact, a decrease that is not solely attributable to a significantly lower MR (Quinlan and Lighton, 1999).

Previous studies have used decapitation, decerebration and anaesthetisation to elicit DGCs in cockroaches (Edwards and Miller, 1986; Matthews and White, 2011b), ants (Duncan and Newton, 2000; Lighton, 1992; Lighton et al., 1993; Quinlan and Lighton, 1999) and moth pupae (Ito, 1954; Levy and Schneiderman, 1966). While it has been acknowledged that decerebration may alter the interactions between respiratory pattern generators, and so alter the behaviour of the DGCs produced by decapitated individuals (Quinlan and Lighton, 1999), the primary cause underlying the emergence of DGCs in decerebrated insects was believed to be their

quiescence and low MR, a state that is not significantly different to that exhibited by resting individuals spontaneously displaying DGCs (Lighton et al., 1993; Lighton and Garrigan, 1995; Quinlan and Lighton, 1999). By reversibly inactivating the insect's brains, we have demonstrated that DGCs can emerge due to a decrease in the activity of the insect's nervous system. Brain inactivation is sufficient to cause the evenly spaced abdominal ventilatory movements typical of continuous gas exchange to become tightly clustered together into the discrete gas exchange periods characteristic of the DGC (Fig. 5A). One possible factor underlying this transformation may be a change in the sensitivity of respiratory chemoreceptors to hypoxic or hypercapnic stimuli in the absence of an active brain. A decreased sensitivity to CO_2 is a distinct possibility, especially in decapitated insects, as studies on the locust indicate that low threshold CO_2 receptors may lie in or near the brain in locusts (Miller, 1960). Some vertebrates show changes in respiratory sensitivity associated with discontinuous breathing [e.g. ground squirrels show a depressed response to hypoxia and an elevated response to hypercapnia when breathing intermittently during hibernation (McArthur and Milsom, 1991)] and similar changes may also occur in insects displaying DGCs. Indeed, this response is seen in decerebrated insects that show decreased sensitivity to both hypoxia (Edwards and Miller, 1986) and hypercapnia (Matthews and White, 2011b; Miller, 1960; Myers and Retzlaff, 1963). However, similar changes have not been observed in intact insects showing DGCs. Intact locusts breathing discontinuously are largely insensitive to hypoxia (11% O_2) (Harrison et al., 1995; Matthews et al., 2012), while remaining sensitive to 2% CO_2 (Harrison et al., 1995). These responses are consistent with the respiratory sensitivity of active, continuously breathing cockroaches, which hyperventilate when exposed to >1% CO_2 and <10% O_2 (Matthews and White, 2011b). Thus, investigating the differences between naturally occurring and artificially induced DGCs will be necessary to determine whether
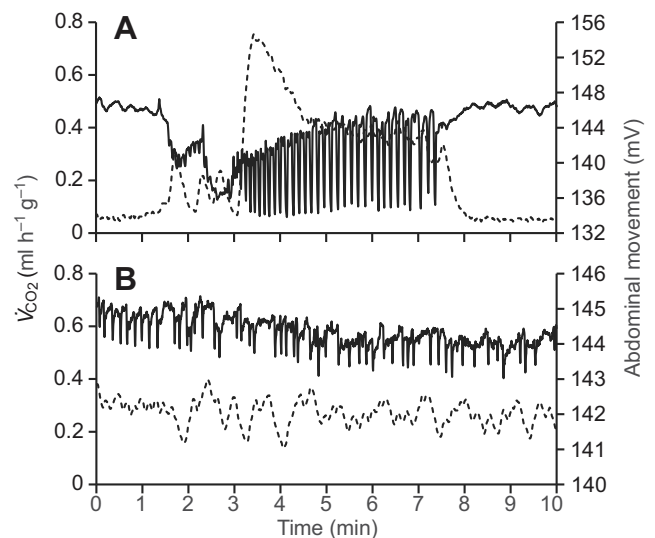

Fig. 5. Examples of abdominal ventilatory movements (solid line) and gas exchange pattern (dashed line) during (A) a single ventilatory period during a discontinuous gas exchange cycle (DGC) while the cold probe was chilled to –0.6°C and (B) during continuous gas exchange after rewarming the head to 25°C. Traces were recorded from the same individual (577 mg male). Downward spikes in voltage indicate the downward flexure of the abdomen towards the infrared movement sensor and are a qualitative indication of abdominal activity.

artificially induced DGCs are functionally identical to the pattern displayed by intact insects.

The clustering of abdominal pulsations into discrete ventilatory episodes during DGCs is reminiscent of the emergence of breath-clustering or episodic breathing patterns displayed by many vertebrates. While mammalian breathing patterns tend to be regular and continuous when they are active, some mammals switch to a breathing pattern consisting of alternating periods of ventilation and breath-holding during hibernation or sleep (McArthur and Milsom, 1991; Milsom et al., 1996). In vertebrate ectotherms, particularly, these periodic or episodic respiratory patterns appear to be intrinsic to the central nervous system (Douse and Mitchell, 1990; Kinkead et al., 1994). This similarity between the discontinuous gas exchange patterns of insects and vertebrates is even more curious when one considers that the respiratory apparatus of these two groups evolved independently, with the muscles and neural circuits used by insects for ventilation belonging to the thorax and abdomen [predominantly involving the active raising and lowering of the abdominal sterna, and longitudinal telescoping of the abdomen (Miller, 1960)] while those used by vertebrates are associated with the buccal cavity or pharynx, and are innervated by neural circuits derived from those that control feeding movements (Milsom, 2010). The similar respiratory patterns of these two very different groups may hint at common factors constraining the evolution of respiratory systems, or perhaps may simply reflect the limited number of ways in which respiratory rhythms may be produced by pattern generators.

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