Respiration Physiology (1988) **73**, 243–256
Elsevier

RSP 01427

Effect of denervation of carotid labyrinths on breathing in unrestrained *Xenopus laevis*

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(Accepted for publication 1 March 1988)

Abstract. The effect of denervation of the carotid labyrinths on breathing responses to simultaneously applied aerial and aquatic normoxia, hypoxia, or hypercapnia has been studied in unrestrained *Xenopus laevis*. Denervation significantly reduced $\dot{V}_I$ of normoxic toads compared with $\dot{V}_I$ in intact and sham-operated toads, due to a significant reduction in the volume of each buccal pumping movement (V_B) in denervates. Breathing increased significantly in response to environmental hypoxia or hypercapnia in intact and sham-operated toads as well as in denervates. Breathing frequency (f_{RESP}) was the major determinant of the increase in $\dot{V}_I$ for V_B was unchanged and even fell slightly in denervates in hypercapnia. Dive time (DT) was significantly reduced in both hypoxia and hypercapnia, from that in normoxia. DT fell significantly more in hypoxia than in hypercapnia in both denervates and intact and sham-operated toads. It is concluded that the carotid labyrinth does not play a major role in regulating breathing in hypoxia or hypercapnia in unrestrained *Xenopus*.

Amphibian; Buccal pump; Chemoreceptor; Dive time; Diving; Ventilation

The carotid labyrinth of anuran amphibians is a cavernous expansion of the common carotid artery enclosing a complex vascular network forming the root of both the internal and external carotid arteries. The functional significance of this vascular network is unknown although the intervascular stroma contains associations of glomus, sustentacular and nerve cells which are presumed to be involved in intravascular chemoreception (Rogers, 1966; Ishii and Ishii, 1976; Ishii and Kasakabe, 1982). Denervation of this region prevents hyperpnea in response to breathing low oxygen (Smyth, 1939) while perfusing the labyrinth of the toad, *Bufo vulgaris*, with hypoxic solutions increases the frequency of afferent action potentials in that branch of the glossopharyngeal nerve innervating the labyrinth and also stimulates breathing (Ishii *et al.*, 1966).

Although the carotid labyrinth has been shown to acutely affect breathing in anurans, the role of the labyrinth in controlling the normal breathing pattern of anurans has not

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been investigated. In most terrestrial, and virtually all aquatic, amphibians lung ventilation occurs intermittently. In more terrestrial species of frogs and toads, the buccal cavity is continuously ventilated between short bouts of pulmonary ventilation (West and Jones, 1975; de Jongh and Gans, 1969). Even so, the pattern is quite labile and can be greatly modified by disturbance or other factors increasing metabolic demand. The elective nature of the control of breathing episodes is exemplified by aquatic anurans. If the surface poses some threat, ventilation only occurs in brief visits to the surface which interrupt maintained periods of submergence (Boutilier, 1984). Metabolism can be maintained by these periodic air breaths as a supplement to cutaneous exchange. Respiratory homeostasis no longer exists and the control systems allow broad fluctuations in all the respiratory variables (Boutilier and Shelton, 1986b; Shelton *et al.*, 1986). At one extreme (*i.e.* forced submergence) even metabolism may be supplemented by anaerobiosis (Jones, 1972; Boutilier and Shelton, 1986a). Hence, the role of peripheral chemoreceptors in intermittent breathers is likely to be quite different from their role in birds and mammals (Jones and Milsom, 1982; Shelton *et al.*, 1986).

Given this, we embarked on these experiments in an attempt to determine the role, if any, of the carotid labyrinth of *Xenopus laevis* in regulating breathing during exposure to simultaneously applied aquatic and aerial normoxia, hypoxia or hypercapnia. Experiments were done on confined, but unrestrained, toads before and some time after sham operations or operations to denervate the carotid labyrinths. We chose *Xenopus* because it offers a couple of advantages when studying the regulation of pulmonary ventilation. First, its buccal force pump is used solely for the purpose of lung ventilation, thereby eliminating the need to differentiate between buccal movements that ventilate only the buccal cavity and those ventilating buccal cavity and lungs (Brett and Shelton, 1979). Second, its aquatic habit means that lung inflation and deflation can be monitored in animals that are confined but unrestrained, by using modified plethysmographic techniques (Lomholt and Johansen, 1974).

Methods

Experiments were done on 14 ♀ *Xenopus* varying in mass from 60 to 64 g. All animals were kept in large aquaria, under constant illumination, in a temperature-controlled room at 25 ± 1 °C. Each toad was fed 4–8 g of beef heart in small pieces every week. Breathing data were obtained from 4 intact (80 h recording), 4 sham-operated (216 h recording), and 6 carotid labyrinth denervated toads (280 h recording).

Denervation of the carotid labyrinths was done on animals surgically anaesthetised by immersion in 1–5% (w/v) MS222 solution. Before and after surgery the animals were immersed for 30 sec in a tetracycline solution to reduce the likelihood of infection. The skin was opened by a ventral midline incision from the sternum to within 0.5 cm of the tip of the lower jaw. The sternum was divided, as close to the mid-line as possible, and the two halves were held apart by retractors to expose the carotid labyrinths. All fat and

nerve tissues surrounding the labyrinths were removed and the blood vessels leading to and from, as well as the labyrinths themselves, were immersed in 95% ethanol for 30–60 sec to ensure complete destruction of all nervous tissues. Cotton swabs, coated with petroleum jelly, were used to form a well around the labyrinth so that ethanol did not damage surrounding tissues. For sham operations the labyrinths were identified and exposed and 0.9% NaCl solution was placed in the well instead of alcohol. Plain gut sutures, running from muscle or connective tissue insertions, were used to close the sternum while the skin was closed using silk sutures. The toads were allowed at least 8 days for recovery from surgery. No differences in breathing responses were noticed in toads which had been denervated between 11 and 179 days. The efficacy of denervation was checked histologically. Animals were killed at the completion of experiments by prolonged immersion in 5% (w/v) MS222 solution. Both carotid labyrinths were removed, fixed in Bouin's solution, embedded in wax and sectioned at 8–12 μm. The sections were stained with Luxol fast blue and counterstained with neutral red.

Each animal was placed in a 1 L, brown, opaque plastic flask 9 cm diameter and 18 cm in height to the base of the neck. The neck was of 2.5 cm uniform diameter and 4 cm in height. The flask was cut into two and, after introducing the animal, the two portions were rejoined using a rubber band cut from a motorcycle inner tube (fig. 1). The flask was filled with water to the middle of the neck, as indicated by the water level indicator

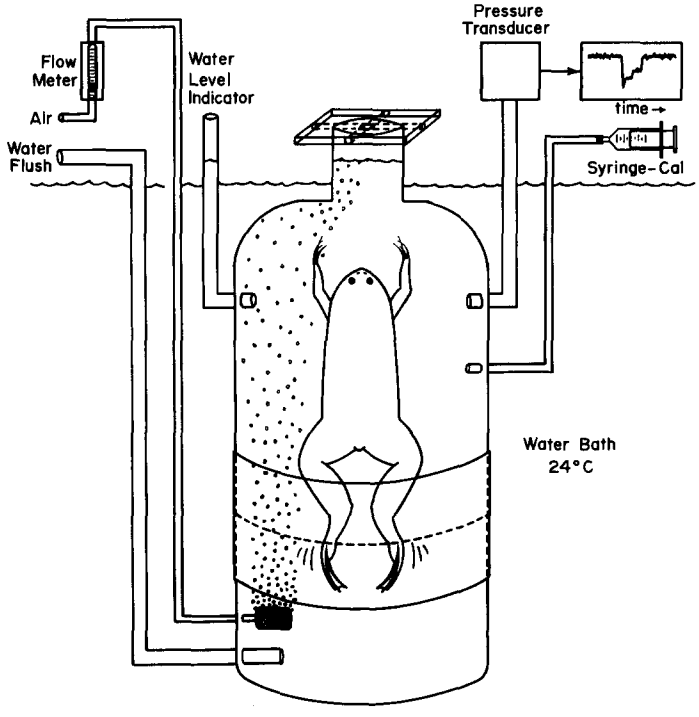

Fig. 1. Diagram of experimental apparatus; see text for further details.

(fig. 1). A 5 cm square piece of black perspex, weighted with brass weights, was placed on the top of the neck to prevent the animals from climbing out. Small grooves were etched in the perspex to allow gas to escape from the flask. Gases were bubbled through the water from a very fine pore air stone located in the bottom of the flask. A constant flow of compressed air of 30 ml · min^{-1} was maintained throughout the period the toad was in the flask. During an experiment gases were obtained from non-analyzed tanks and were pumped into the flask by precision gas mixing pumps (Wösthoff, Bochum, F.R.G.). Water samples were taken into a syringe connected to the middle of the flask by P.E. 190 polyethylene tubing (Clay Adams, B & D Inc., Parsippany, NJ, U.S.A.) (fig. 1). Gas samples were taken from the air-space in the neck. The gas sampling syringe was connected to the neck by PE 100 polyethylene tubing. The water and gas samples were analyzed for P_{O_2} and P_{CO_2} using an Instrumentation Laboratory pH/blood gas analyzer Model 13 with electrodes thermostated at 24 °C (Instrumentation Laboratories Inc., Lexington, MA, U.S.A.). The electrodes were calibrated with humidified, precision gas mixtures of 1% CO_2 in air and 2 or 3% CO_2 in N_2.

Each flask was connected via PE 190 tubing to a Statham Physiological pressure transducer (fig. 1). The models used in these experiments were P23Db, P23BB and P23V (Statham Laboratories, Inc., Hato Rey, Puerto Rico). The transducer measured the change in water level in the neck of the flask caused by the exhalations and inhalations of a toad. The change in water level was linearly proportional to the volume of air breathed in or out over a range of ± 10 ml. Each flask was calibrated by injecting and withdrawing a measured volume of water using the water sampling syringe (fig. 1). The signals from the transducers were amplified and displayed on Fisher Recordall Series 5000, dual pen, strip chart recorders (Fisher Scientific, Ottawa, Ontario, Canada).

Observations were made on 4 toads simultaneously. The flasks containing the toads were placed in a water bath at 24 ± 1 °C. The temperature of the bath was continuously recorded by a digital thermometer connected to a potentiometric recorder. Electric lights in the room were continuously on. Intact, sham operated and denervated toads were assigned to the flasks at random with the proviso that at least one denervated animal was included in every experiment. No recordings were made for the first 3 days after the animals were placed in the experimental apparatus as we found that this was the time required to obtain a stable breathing pattern in air equilibrated water. Over the next 10 to 15 days toads were subjected to various combinations of O_2 and CO_2. The gas combinations were restricted to P_{O_2} in the range of 157 to 22 mm Hg at low P_{CO_2} values (1.7–4.6 mm Hg) and P_{CO_2} in the range of 1.9–22.5 mm Hg with P_{O_2} held close to air saturation values. Three hours were allowed for gas equilibration in the flask and breathing movements were recorded for 6–10 h after this period. Two different gas mixtures were used in any one experiment, one gas mixture being applied to a pair of flasks. P_{O_2} and P_{CO_2} in air and water were monitored frequently during the observation period. Gases in air and water were the same and remained constant after the 3 h equilibration period. At the end of a recording period the gas mixture was replaced by air. Every other day each flask was flushed with fresh 24 °C water, at 20–30 ml · min^{-1},

from a header tank for 60 min (fig. 1). The minimum time between test runs was 12 h and the maximum 60 h.

Xenopus exhales when it first comes to the surface and the lungs are then inflated with one, two or, at most, three pumps of the buccal cavity. The volume represented by each single buccal pump in lung inflation (V_B) was measured on the recorder charts using an HP model 9111A graphics tablet (Hewlett Packard Inc., Palo Alto, CA, U.S.A.). These values were stored in a computer and a single mean value for V_B was obtained for the 6–10 h run. The mean value was expressed as mass-specific V_B ($ml \cdot 100\ g^{-1}$). The total number of buccal movements were counted in each hour of a run and averaged to give f_{RESP} ($frequency \cdot h^{-1}$). Total inhaled volume ($\dot{V}_I$) of an individual animal was obtained as the product of the mean mass specific buccal volume and mean frequency per hour. The time spent underwater, between breathing periods at the surface, was designated as the dive time (DT). Total DT was determined over the 6–10 h recording period and expressed as $min \cdot h^{-1}$. To differentiate unequivocally between pauses between breaths in a breathing burst or bout and genuine periods of submergence, intervals between breaths of less than 5 min were not included in determining DT (Boutilier, 1984). Four single values (V_B, f_{RESP}, $\dot{V}_I$ and DT) from each run were stored in the U.B.C.-M.T.S. mainframe computer and values from all experiments were displayed as three-dimensional graphs using the 'Disspla' program (Integrated Software Systems Corp., San Diego, CA, U.S.A.). Values of variables monitored over restricted ranges of P_{O_2} and P_{CO_2} were also averaged and are given in the text as means $\pm$ 1 SD. Air mean values (normoxia) were compared with those obtained with P_{O_2} in the range of 35–65 mm Hg (hypoxia) and P_{CO_2} in the range of 11–22.5 mm Hg (hypercapnia; P_{O_2} at air saturation) using an unpaired two-tailed *t*-test. The unpaired two-tailed *t*-test was also used to compare mean values between denervated and intact and sham operated toads. In all cases, $P < 0.1$ was taken as the fiducial limit of significance.

Results

At air saturation, mean values for V_B ($3.45 \pm 0.58\ ml \cdot 100\ g^{-1}$) and $\dot{V}_I$ ($42.8 \pm 17\ ml \cdot 100\ g^{-1} \cdot h^{-1}$) in sham-operated and intact *Xenopus* were significantly above those in carotid labyrinth denervates ($V_B = 2.64 \pm 0.21\ ml \cdot 100\ g^{-1}$ and $\dot{V}_I = 23.1 \pm 5.6\ ml \cdot 100\ g^{-1} \cdot h^{-1}$). Breathing frequency was also lower in denervates ($8.55 \pm 1.8 \cdot h^{-1}$) compared with intacts and shams ($12.3 \pm 3.5 \cdot h^{-1}$), but this difference was not significant. There was no significant difference between DT of intacts and shams ($51.3 \pm 4.9\ min \cdot h^{-1}$) and denervates ($48.9 \pm 1\ min \cdot h^{-1}$) in normoxia.

Exposure to low oxygen or elevated CO_2 stimulated breathing in denervated and sham-operated and intact toads (figs. 2 and 4). Breathing frequency was extremely sensitive to hypoxia or hypercapnia. In intacts and shams, f_{RESP} increased significantly from $12.3 \pm 3.5 \cdot h^{-1}$ in normoxia to $48.8 \pm 11 \cdot h^{-1}$ when P_{O_2} was in the range of 35–65 mm Hg (hypoxia). A similar significant increase in f_{RESP} occurred when P_{CO_2} was increased to 11–22.5 mm Hg while P_{O_2} was held close to air saturation

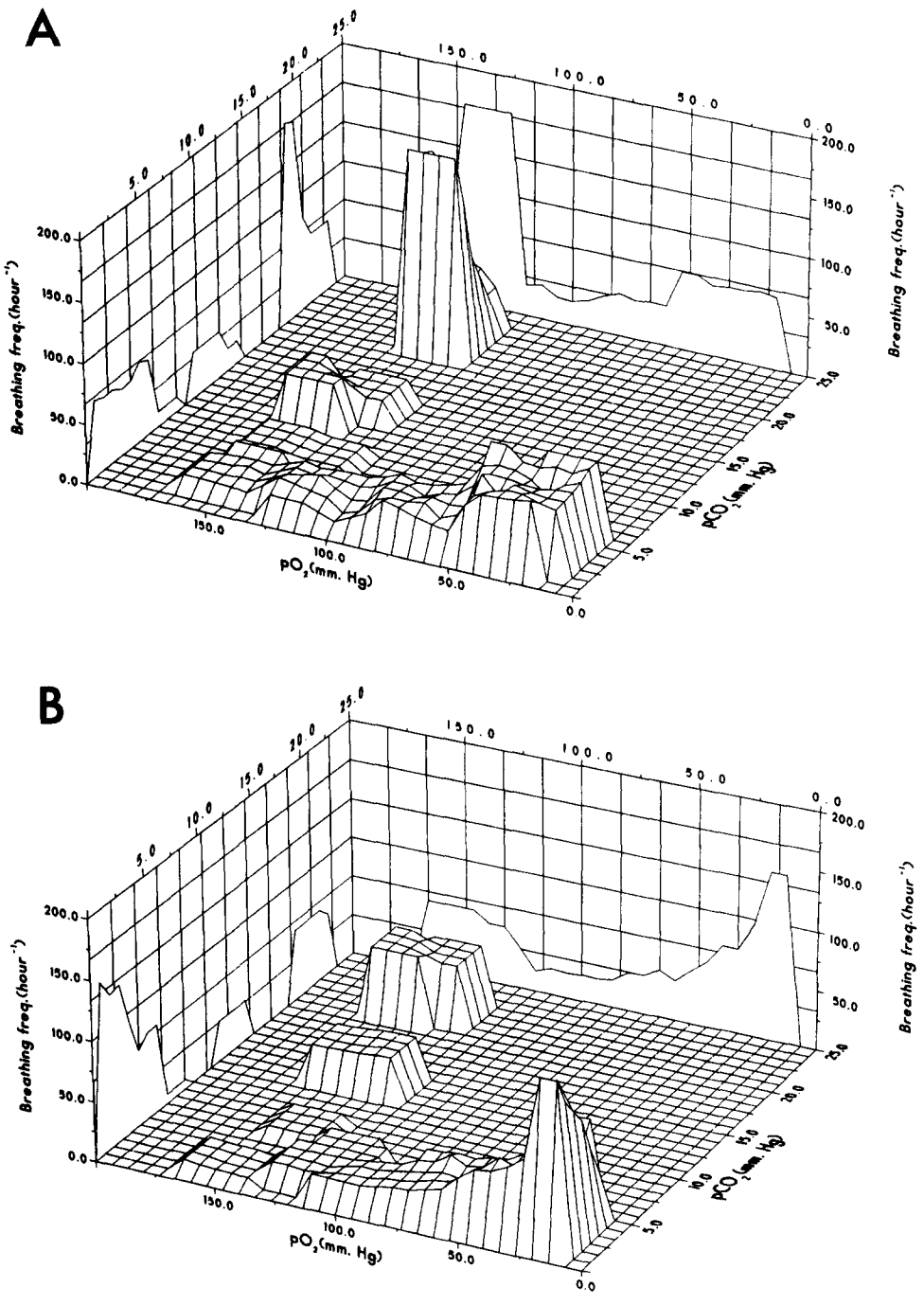

Fig. 2. Three dimensional plot of breathing frequencies ($f_{RESP} \cdot h^{-1}$; z-axis) engendered by decreases in aerial and aquatic P_{O_2} (x-axis) and increases in aerial and aquatic P_{CO_2} (y-axis) in normal and sham operated (A) and denervated (B) toads.

(hypercapnia). In denervates, f_{RESP} increased significantly from $8.5 \pm 1.8 \cdot h^{-1}$ in normoxia to $43.7 \pm 13.9 \cdot h^{-1}$ in hypoxia. In hypercapnia, f_{RESP} increased significantly to $46.0 \pm 12.1 \cdot h^{-1}$. There were no significant differences between the response of f_{RESP} in denervates and intact and sham-operated toads in hypoxia or hypercapnia (fig. 2a,b).

V_B was unaffected by decreases in environmental P_{O_2} or increases in P_{CO_2} except in denervates in which V_B fell significantly from 2.64 ± 0.21 ml $\cdot$ 100 g^{-1} (at air saturation) to 2.1 ± 0.1 ml $\cdot$ 100 g^{-1} in hypercapnia (fig. 3a,b). V_B of 2.1 ± 0.1 ml $\cdot$ 100 g^{-1} in hypercapnic denervates was significantly below that in hypercapnic intact and sham-operated *Xenopus* (3.15 ± 0.39 ml $\cdot$ 100 g^{-1}).

In hypoxia f_{RESP} increased, while V_B was unaltered, so that $\dot{V}_I$ followed changes in f_{RESP} (fig. 4a,b). In hypoxia, $\dot{V}_I$ increased significantly from 42.8 ± 17 (in normoxia) to 133.9 ± 11 ml $\cdot$ 100 $g^{-1} \cdot h^{-1}$ in intacts and shams while in denervates it increased significantly from 23.1 ± 5.6 (in normoxia) to 133.6 ± 64 ml $\cdot$ 100 $g^{-1} \cdot h^{-1}$ (fig. 4a,b). Hypercapnia caused $\dot{V}_I$ to increase significantly to 93 ± 25 ml $\cdot$ 100 $g^{-1} \cdot h^{-1}$ in denervates, despite the significant decline in V_B, and to 113.2 ± 61 ml $\cdot$ 100 $g^{-1} \cdot h^{-1}$ in intacts and shams (fig. 4a,b).

DT was significantly reduced by exposure to hypoxia or hypercapnia compared with DT in normoxia (fig. 5a,b). DT fell much more in hypoxia (to 30% of normoxic DT) than hypercapnia (to 70% of normoxic DT) in all animals and was the only variable that was affected differently by these two gaseous environments. DT in hypoxia (intact and sham = 14 ± 14 min $\cdot h^{-1}$; denervate = 14.3 ± 5.7 min $\cdot h^{-1}$) was significantly shorter than DT in hypercapnia (intact and sham = 34.4 ± 12.1 min $\cdot h^{-1}$; denervate = 34.8 ± 9.9 min $\cdot h^{-1}$) in both denervates and intact and sham-operated toads. However, there were no significant differences between the effects of hypoxia or hypercapnia on DT in denervated and intact and sham-operated toads (fig. 5a,b).

Histological preparations of the labyrinths of intact and sham-operated toads showed nerve fibres running in the adipose tissue surrounding the glands. In contrast, no nerve fibres were seen in denervates. In fact, ethanol exposure had considerably disrupted the adventitial layers of the labyrinths and carotid arteries in denervates, which was not observed in the shams in which saline was applied to the labyrinths. These sections convinced us that *no* neural innervation remained in the denervates.

Discussion

The present experiments have shown that exposure to environmental hypoxia or hypercapnia caused a substantial increase in $\dot{V}_I$ in *X. laevis*. Furthermore, this increase in $\dot{V}_I$ was unaffected by denervation of the carotid labyrinths. In hypoxia or hypercapnia, $\dot{V}_I$ increased about 4 times in intact and sham-operated frogs while for denervates $\dot{V}_I$ increased 4 times during hypercapnia, but went up nearly 6 times in response to hypoxia. Despite this difference in relative change, $\dot{V}_I$ in denervates was the same as $\dot{V}_I$ in intact and sham-operated toads at P_{O_2} between 35 and 65 mm Hg. These

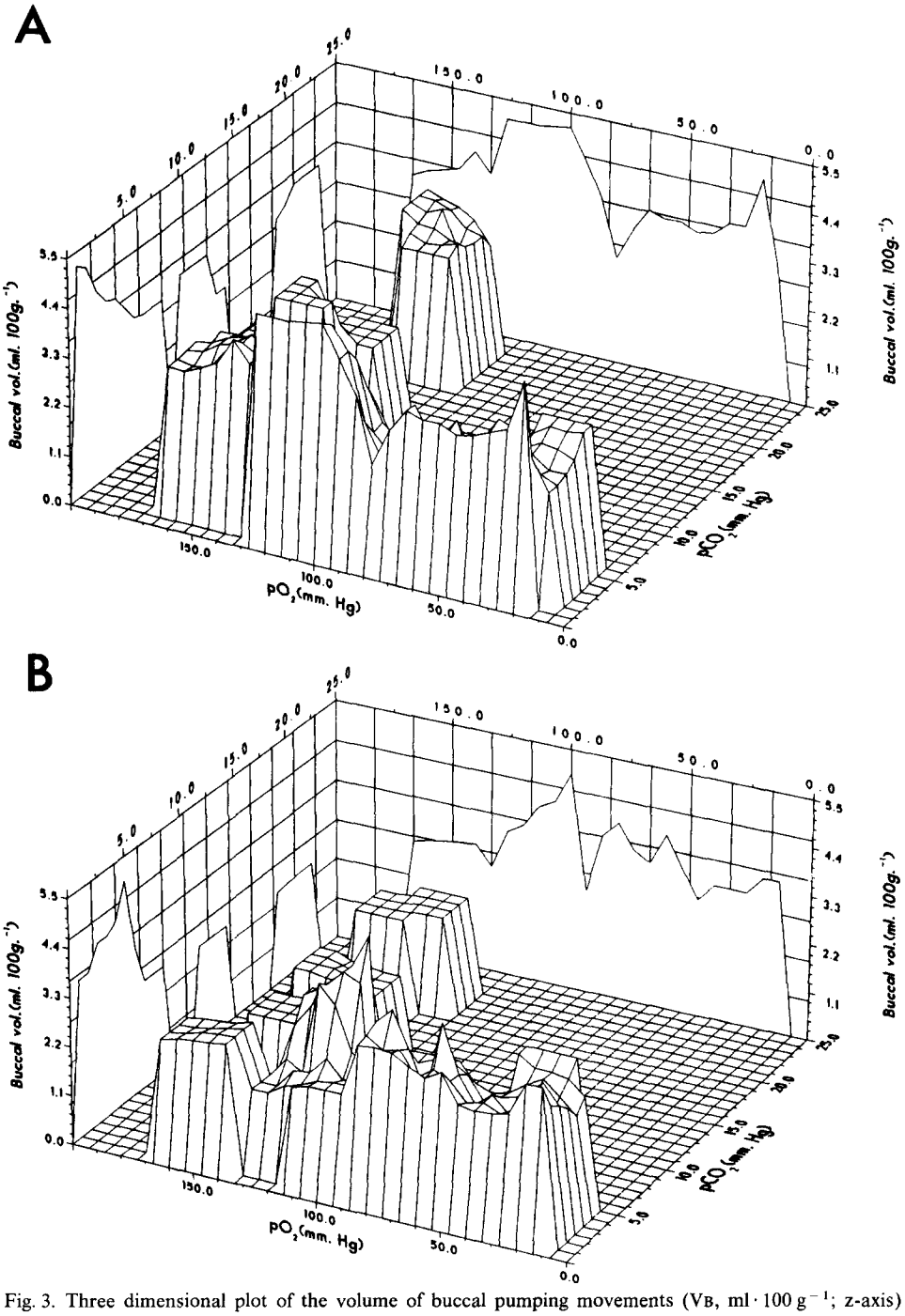

Fig. 3. Three dimensional plot of the volume of buccal pumping movements (V_B, $ml \cdot 100\,g^{-1}$; z-axis) engendered by decreases in aerial and aquatic P_{O_2} (x-axis) and increases in aerial and aquatic P_{CO_2} (y-axis) in normal and sham operated (A) and denervated (B) toads.

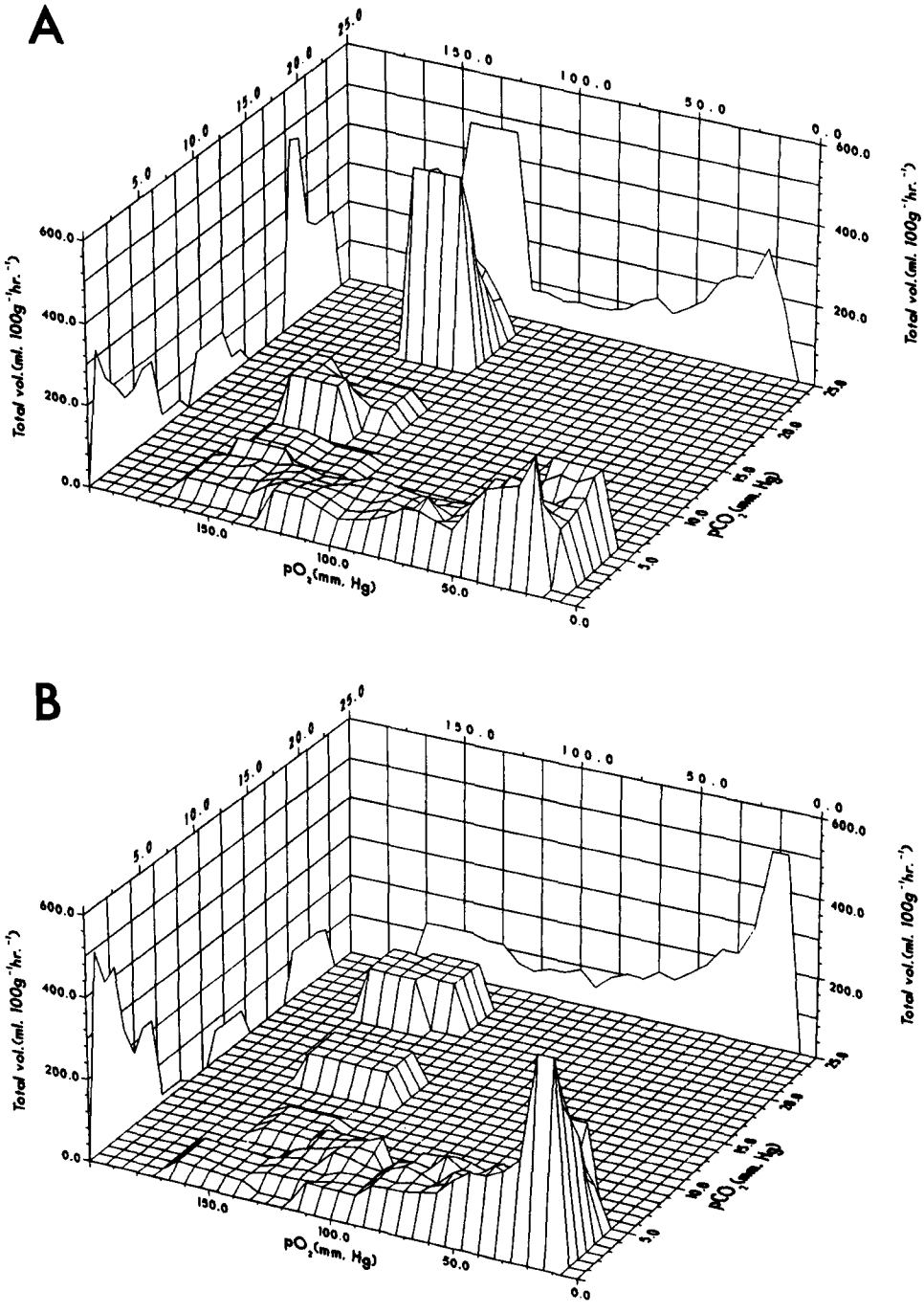

Fig. 4. Three dimensional plot of total volume inhaled ($\dot{V}_I$, ml · 100 $g^{-1} \cdot h^{-1}$; z-axis) engendered by decreases in aerial and aquatic P_{O_2} (x-axis) and increases in aerial and aquatic P_{CO_2} (y-axis) in normal and sham operated (A) and denervated (B) toads.

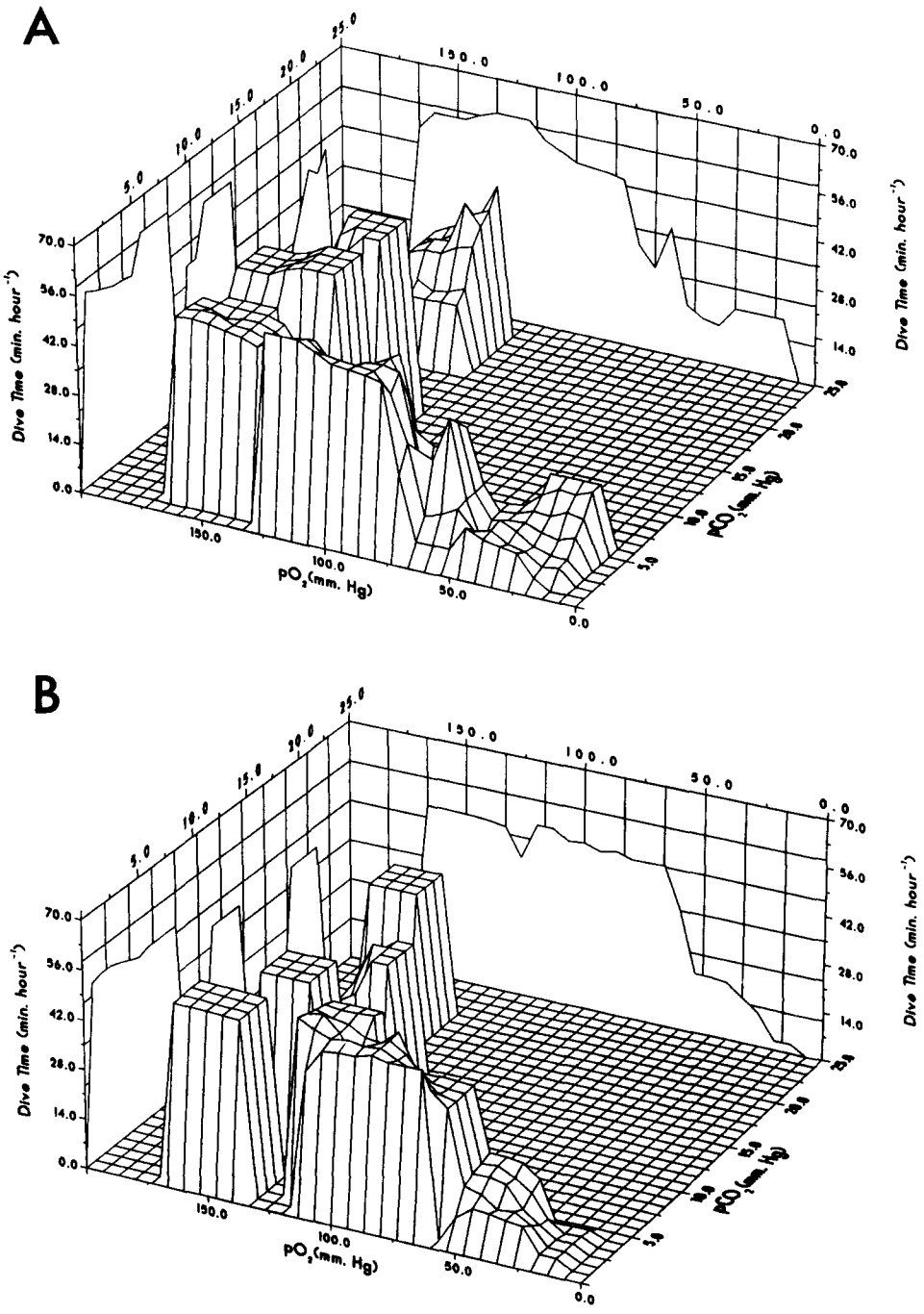

Fig. 5. Three dimensional plot of dive times (DT, $min \cdot h^{-1}$; z-axis) engendered by decreases in aerial and aquatic P_{O_2} (x-axis) and increases in aerial and aquatic P_{CO_2} (y-axis) in normal and sham operated (A) and denervated (B) toads.

observations confirm and extend those of Evans and Shelton (1984) who reported that denervation of the carotid labyrinths in *Xenopus* did not affect the proportionate increase in ventilation in response to hypoxia. This raises the question as to whether this is unique to *Xenopus*, for although the microscopical anatomy of the labyrinth in *X. laevis* is quite similar to that in the toad, *B. vulgaris* (Ishii and Kusakabe, 1982), the role of the labyrinth in acutely affecting breathing in *B. vulgaris* and other anurans is well established (Smyth, 1939; Ishii *et al.*, 1966; Lillo, 1980).

Nevertheless, there was an indication that the labyrinth exerts some control on eupnea in *Xenopus* in that $\dot{V}_I$ of denervates was about half that of intacts and shams in normoxia. Denervation reduced both V_B and f_{RESP} although only the former was reduced significantly. The combined effect was a significant fall in $\dot{V}_I$ after denervation. Evans and Shelton (1984) also reported that lung ventilation fell in normoxia after denervation of the labyrinths due to decreases in both frequency and volume of inspirations. Birds and mammals, in which the role of carotid chemoreceptors in controlling breathing responses to hypoxia is well established, also show a decline in normoxic ventilation after chronic carotid chemoreceptor denervation (Bouverot, 1978; Lillo and Jones, 1982; Fitzgerald and Lahiri, 1986).

Chemoreceptive sites for oxygen are widely dispersed throughout the central cardiovascular system of lower vertebrates (Muratori, 1934). The present data implies that sites located in the pulmonary or aortic regions may be the receptive limb of the oxygen chemoreflex in anuran amphibians (Ishii *et al.*, 1985). Alternatively, it is possible that there is a central oxygen sensor which may be the functional analogue of a central oxygen sensor which has been demonstrated in turtles (Hitzig and Nattie, 1982). There is some evidence to suggest that anurans also have central chemoreceptors sensitive to CO_2 and $[H^+]$ (de Marneffe-Foulon, 1962). This raises the question as to whether some of the hypoxic ventilatory response can be attributed to these central receptors, stimulated by changes in acid–base balance caused by anaerobiosis. Boutilier and Shelton (1986a), however, have shown that anaerobiosis does not occur in voluntarily diving *Xenopus* while arterial P_{O_2} is above 15–20 mm Hg. Hence, although acid–base changes could contribute to increased $\dot{V}_I$ at the lowest environmental O_2 levels experienced by our toads (22 mm Hg) substantial increases in $\dot{V}_I$ occurred when environmental P_{O_2} was around 50 mm Hg, even in denervates, when blood oxygen tensions would be expected to far exceed the critical level for anaerobiosis in voluntary dives (Boutilier and Shelton, 1986a).

Recently, Boutilier (1984) presented a detailed analysis of breathing in voluntarily diving *Xenopus*. Breathing was differentiated into bouts and bursts and the onset and termination of breathing periods was analysed using log-survivor plots (Fagen and Young, 1978). Boutilier (1984) concluded that the major variable in the control of breathing was not $\dot{V}_I$ and f_{RESP} but the length of the period between breaths. Similar conclusions have been reached from studies on other intermittent breathers (Milsom and Jones, 1980). This raises the question as to whether our present conclusions would be any different if stress had been laid on the non-breathing rather than breathing periods. DT declined significantly in hypoxia and in hypercapnia in both the sham and

intact group as well as in denervates. However, in all toads, DT was significantly longer in hypercapnia than hypoxia. In the absence of any measure other than dive time our conclusion about the effect of hypoxia compared with hypercapnia on breathing would be changed (*i.e.* change in breathing pattern) but not with respect to the effects of denervation. Given that a change in f_{RESP} and not buccal volume was the major contributor to increased $\dot{V}_I$ in hypoxia, and in hypercapnia, then it appears that measurement of breathing frequency, or its reciprocal the non-ventilatory period, will both give a similar measure of respiratory responses to these environmental conditions. Consequently, our conclusions concerning the lack of any major role for the carotid labyrinth in regulating breathing in hypoxia and hypercapnia in behaving *Xenopus* will be correct regardless of the approach taken in data analysis.

Acknowledgements. We are grateful to Dr. R. Boutilier for helpful discussions at the start of the project and to Dr. W.K. Milsom and Michael Hedrick for comments on the first draft of the manuscript. The research was supported by operating and equipment grants from NSERCC to D.R.J.

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