

Contribution of the diaphragmaticus muscle to vital capacity in fasting and post-prandial American alligators (*Alligator mississippiensis*)

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Summary

The importance of the diaphragmaticus muscle to vital capacity was investigated in juvenile American alligators by transection of this muscle. In both fasting and post-prandial animals a pneumotach was used to study vital capacity that was stimulated by either a hypercapnic–anoxic gas mixture or a hypercapnic–normoxic gas mixture in two types of control groups of animals (a sham-operated group and a group receiving no treatment) and in the experimental (transected) group. Transection did not significantly reduce vital capacity or affect time of inspiration or expiration in fasted animals. For both the experimental and control groups vital capacity was greatly reduced in post-prandial animals compared to the fasting state. Furthermore, alligators with a transected diaphragmaticus muscle showed a 16–18% greater drop in

vital capacity in the post-prandial state than did alligators with an intact diaphragmaticus muscle. The post-prandial decrease in vital capacity for alligators with a transected diaphragmaticus occurred concomitantly with a significant increase in time to inspire and a decrease in maximum rate of inspiration when compared to control animals. The results from this study suggest that the diaphragmaticus muscle plays an important role in enabling large volumes of oxygen to be taken into the lungs in the post-prandial state.

Key words: alligator, *Alligator mississippiensis*, CO₂ breathing, diaphragmaticus, ventilation, post-prandial state, vital capacity, evolution, archosaur, ventilatory mechanics, crocodylian.

Introduction

The breathing apparatus of crocodylians is tripartite, consisting of costal ventilation, in which the ribs and intercostal muscles change the volume of the thorax producing inspiration and expiration (Gans and Clark, 1976), and two mechanisms that supplement inspiration (Fig. 1). The first mechanism, the hepatic piston, entails a decrease in pleural pressure caused by a caudal movement of the lungs, liver and other viscera (Boelert, 1942; Gans and Clark, 1976). Pelvic aspiration refers to an increase in abdominal volume and decrease in abdominal pressure caused by rotation of the pubes and gastralia caudoventrad (Farmer and Carrier, 2000). An important element of the hepatic piston is the diaphragmaticus muscle. This is a pair of two thin muscles extending in a caudocranial orientation (Reese, 1915); the lateral component of the muscle is attached by a short tendon in front of the hip joint to the edge of the ilium, and the ventral portion originates partly on the innermost side of the last (caudad) gastralia, which is embedded in the rectus abdominus muscle [American alligator (Farmer and Carrier, 2000)] and partly on the border of the pubis [caiman (Gans and Clark, 1976; Reese, 1915)]. The diaphragmaticus fans as it runs cranially to its place of insertion. Ventrally and laterally

the muscle inserts directly on the pericardium and on a fibrous membrane that encapsulates the liver. Dorsally the muscle inserts on an aponeurosis that passes over the dorsal border of the liver and that binds the liver to the esophagus in the region of the lower esophageal sphincter (Reese, 1915). Unlike the mammalian diaphragm which partitions the body cavity obliquely into thoracic and abdominal chambers, the crocodylian diaphragmaticus encases laterally and ventrally most of the peritoneal viscera. Because the lungs adhere to the hepatic membrane, contraction of the diaphragmaticus pulls the liver–lung complex caudad and facilitates inspiration (Gans and Clark, 1976).

Although relatively rare, a few other groups of tetrapods have evolved similar specialized muscles: amphibians of the family Pipidae (*Xenopus laevis* and *Pipa pipa*) have a muscle complex (musculus pulmonum proprius – ventral diaphragm) that is analogous to the diaphragmaticus of crocodylians. Laterally the musculus pulmonum proprius originates on the edge of the ilium (*Xenopus*) or on the femur (*Pipa*) whereas the ventral diaphragm originates on the deep layers of the rectus abdominus. This muscle complex expands as it runs cranially, and inserts on the dorsal aspect of the lung, the distal esophagus in the region of the lower esophageal sphincter, and

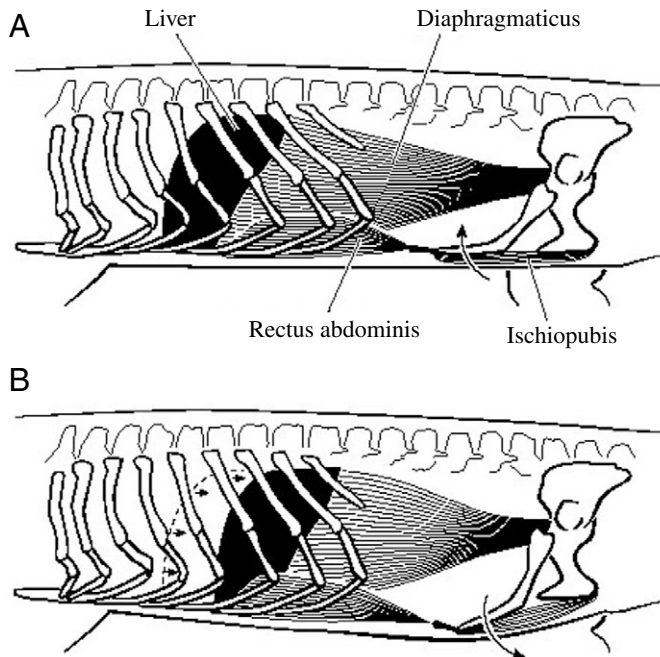


Fig. 1. Illustration of the mechanism of ventilation in crocodylians. (A) Expiration is produced by mediocaudal rotation of the ribs and constriction of the abdominal cavity, which produces a cranial translation of the viscera. Constriction of the abdomen results from activity of the transversus abdominis and the rectus abdominis muscles, which rotate the pubes and gastralia craniodorsad. (B) Inspiration is produced by laterocranial rotation of the ribs and caudal translation of the viscera. The viscera, including the large stomach and liver, are pulled caudad by contraction of the diaphragmaticus muscle. The ischiopubic and ischiotruncus muscles increase the abdominal volume by rotating the pubes and gastralia ventrocaudad. [Figure taken with permission from Farmer and Carrier (Farmer and Carrier, 2000).]

on a fibrous cervical aponeurosis. The ventral diaphragm inserts on the lobes of the liver, lungs, cervical aponeurosis, and the septum transversum. As in crocodylians, this muscle does not separate the lungs from the abdomen (Keith, 1905). Some testudines (e.g. *Lissemys* and *Trionix*) also have a muscle called a diaphragmaticus, which differs because the fibers have dorsoventral orientation. In this group the diaphragmaticus (cranial) together with the transversus abdominis (caudal) enclose the viscera. These muscles arise from the inner lateral border of the carapace in the region of the first to seventh ribs, proceed both laterally and ventrally, and, after becoming membranous, insert on the plastron in the middle and on the dorsal aspect of the pubic symphysis (George and Shah, 1959).

The selective factors that have favored the evolution of these structures in some groups of tetrapods but not in others remain incompletely understood. Insight into these selective factors comes from understanding the relative importance of the muscles to different facets of the life history of the animals. These muscles may facilitate inspiration (Gans and Clark, 1976), expiration (Gaunt and Gans, 1969; Snapper et al., 1974),

gastrointestinal functions (Pickering et al., 2004; Pickering and Jones, 2002), or venous return (Keith, 1905). They may be most important during exercise, while diving, basking, digesting, or at some other time.

American alligators are highly aquatic in their lifestyle and tend to eat large meals. Theoretically, a large mass of food in the stomach could significantly increase the difficulty of inspiring large volumes of air into the lungs. This in turn could impact numerous facets of their life history, such as dive-time and exercise capacities. Thus, we undertook this study of the importance of the diaphragmaticus muscle to vital capacity during the postprandial period in American alligators to contribute to our understanding of when and how this muscle is used.

Materials and methods

Animal care

American alligators (*Alligator mississippiensis* Daudin 1801) were obtained as embryos from the Rockefeller wildlife refuge. After hatching the animals were housed together in 100-gallon cattle troughs with circulating water that was cleansed by sand filters and kept at a temperature of 26°C. The cages contained basking platforms and full-spectrum lights. The animals experienced a 12 h:12 h light:dark cycle. Animals were fed a diet of crickets, goldfish and the hearts and gizzards of chickens. The animals were removed from their holding tanks twice per week and exercised for short periods (approximately five minutes) on a treadmill.

Experimental design

Similar experiments were conducted sequentially on two sets of animals and will be identified from now on as study 1 or study 2. In study 1 very young individuals were used. Shortly after complete absorption of the yolk sac, the animals of study 1 were randomly divided into two groups. The first group served as the control and in animals of the second group the diaphragmaticus muscle was transected. The control animals did not undergo a sham surgery. At the time of the surgery for transection the animals' mass ranged from 49 to 66 g with a mean mass of 54.2 ± 7.3 g. A series of experiments was conducted with these animals that yielded significant differences between the transected and control groups. At the time of these experiments, the animals weighed between 62 to 130 g, with a mean mass of 97.5 ± 18.1 g.

To ensure that these differences were not due to effects of the surgery, other than the transection of the muscle, the experiments were repeated on a second set of animals, study 2. In this study 2 year old animals were randomly divided into two groups. The first group received a sham surgery and in animals of the second group the diaphragmaticus muscle was transected. At the time of the surgery the animals' weight ranged from 112 to 153 g with a mean mass of 128.2 ± 15.3 g. At the time of the experiments, these animals weighed between 97 and 210 g, with a mean mass of 148.2 ± 36.5 g.

Surgery

Animals were lightly anaesthetized by enclosure in a box with a rag soaked in isoflurane. Once drowsy, they were intubated and ventilated with a small animal ventilator (CWE Inc., Ardmore, PA, USA) using air that had passed through an isoflurane vaporizer (Dräger, Lubeck, Germany). The level was initially set at 4%, but was reduced to 0.5% for the majority of the surgery. All surgeries were performed using sterile technique. With the animal in the supine position, the belly and sides were disinfected with betadine and the animal was draped with a sterile cloth. An incision was made along the ventral midline of the animal, caudad to the last long rib. The ventral portion of the diaphragmaticus muscle was located along both the right and left sides of the body. In the experimental animals the muscles were gathered with forceps, cleared of veins and other organs, and transected. Two more incisions were made on each lateral side of the animals, craniad to the pelvic girdle. The pelvic attachment of the diaphragmaticus to the ilium was identified and severed.

In the sham surgeries the same incisions were made and the muscles were identified but not transected. All incisions were sutured. The animals were treated both with a topical antibiotic and intraperitoneal injections of antibiotics (Baytril) until the incisions had healed. During healing, the animals were kept dry and were given water orally every day.

Ventilation

A pneumotach, which also functioned as a mask, was made using 2 cm of a 5 ml syringe. Two ports were drilled into the side to attach polyethylene tubing (PE190). Stainless steel mesh (size 200 mesh) was placed between the two ports. The tubing was then attached to a pressure transducer 30 cm away from the mask. The top of the mask was only partly covered, allowing air to escape the mask and ensuring low resistance of the pneumotach. A third port was drilled at the bottom of the mask to push air up and out of the top of the mask. Air-flow was set by a flow control unit (Bel-Art Products, Pequannock, NJ, USA) at 50 ml min⁻¹. The system was calibrated by injecting known volumes of gas into the mask prior to placement on the animal. The mask was sealed over the nares of the alligator using dental adhesive (Impregum F, 3M ESPE, St Paul, MN, USA).

Data collection and analysis

Analog signals from the pressure transducer (Omega PX 42.3±1.7 kPa; Omega, Stamford, CT, USA) were amplified (0.3 V mV⁻¹) and filtered (10 Hz low pass) using a Grass amplifier (Grass Telefactor, West Warwick, RI, USA). The analog signals were then converted to a digital form using an analog to digital converter (Biopac Systems, Goleta, CA, USA) and stored on a Macintosh computer. Signals were sampled at a rate of 50 Hz and analyzed with Acqknowledge software (Biopac Systems, Goleta, CA, USA). Volume, time and maximum velocity for both inspiration and expiration were then determined. To account for differences in body mass between individuals, the relative vital capacities were

computed by dividing volume by body mass. Statistical significance was checked using a *t*-test with statistical significance set at *P*<0.05.

Experimental protocol

Study 1

These experiments were conducted on control (no surgery) and experimental animals approximately 1 year after the animals underwent surgery. Animals were fasted for 4 days prior to data collection. They were transferred into large Rubbermaid (Rubbermaid Inc., Fairland, OH, USA) containers from their holding tanks and taken to the experimental room at least 2 h before the experiments commenced. The experimental room was kept at 20°C. The order of the animals was randomly chosen for each trial. The tail and front legs were fixed to the table with duct tape in such a way as to allow the animal to stand but not run around on the table. A mask was attached and the ventilation trace checked to ensure the nares were not occluded and that the mask was properly sealed. Ventilation was then stimulated with a gas mixture (5% CO₂, balance N₂) that flowed through the mask for a period of 3 min. The largest single inhalation occurring during this period and the subsequent expiration were selected for analysis. The same protocol was repeated on the animals 24 h after consuming a meal to satiation of the gizzards and hearts of chickens. The control and experimental animals voluntarily consumed an average of 15% of their mass in food, with the meal size varying from 9–23% of the controls' body mass and 9–20% of the experimental animals' body mass. There was no significant difference (*P*>0.05 *t*-test) in the average voluntary meal size consumed between the two groups. The same five control and five experimental animals were used for each treatment.

Study 2

These experiments were conducted on sham operated and experimental animals approximately 1 week, 2 weeks, 2 months and 6 months after the animals underwent surgery. The same protocol for collecting data as in study 1 was followed for this study with the exceptions given below. Ventilation was stimulated with a gas mixture containing 5% CO₂, balance N₂ or 5% CO₂, 21% O₂, balance N₂, which flowed through the mask for a period of 3 min. For the post-prandial portion of the experiment the animals were force-fed an amount of homogenized chicken liver weighing 11% of the alligator's body mass. The same three sham and three experimental animals were used for each treatment.

Results

Study 1: comparison between experimental and control groups

In the fasted state there was no significant (significance *P*≤0.05) difference between the experimental and control groups of animals in the following parameters: maximum inspiratory volume, expiratory volume, inspiratory time, expiratory time. It is in the post-prandial state that a difference

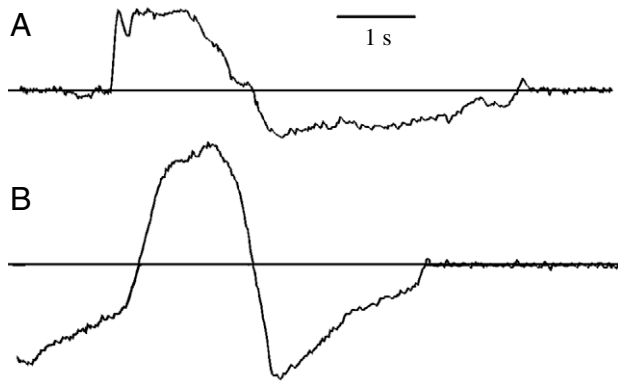


Fig. 2. Sample recordings of ventilation during 5% hypercapnic anoxic treatment from two post-prandial American alligators. (A) Data from an alligator with a transected diaphragmaticus (body mass 93 g). (B) Data from a control alligator (body mass 94 g). A breath cycle normally begins with an exhalation, followed by an inhalation and subsequent apnea. Expiration and inspiration occur when the trace is above and below the solid line (zero marker for airflow), respectively. The beginning of inspiration has been aligned to better illustrate the difference between the animals in both time to inspire and breath size. Calibration bar, 1 s.

in ventilation between the two groups was observed, even though the groups ate equivalent meals. The control and experimental animals voluntarily consumed an average of 15% of their mass in food, with the meal size varying from 9–23% of the controls' body mass and 9–20% of the experimental animals' body mass. There was no significant difference ($P > 0.05$, t -test) in the average voluntary meal size consumed between the two groups. A sample of ventilation data is provided in Fig. 2 illustrating the differences between the experimental and the control groups in a post-prandial state. The maximum inspiratory volume (vital capacity) in the group that underwent transection was significantly less than in the control group (mean 49.6 ml kg^{-1} and 62.2 ml kg^{-1} , respectively; Fig. 3). On average, the experimental group took 2.8 s to inhale the maximum volume, whereas the control group took an average 2.1 s. The control group was able to reach a higher volume in a shorter time by generating higher maximum flow during their breath (Fig. 3). The maximum flow measurements of the control group averaged $3042.2 \text{ ml min}^{-1} \text{ kg}^{-1}$ compared with $1620.0 \text{ ml min}^{-1} \text{ kg}^{-1}$ of the experimental group. There was no significant difference between the maximum rates of exhalation between the two groups.

Study 1: comparison between fed and fasted states

Compared with the fasting state the vital capacity decreased post-prandially for both the experimental and control groups (37.4 ml kg^{-1} and 20.2 ml kg^{-1} , respectively), however inhalation time was not different (Fig. 3). Similarly, compared with the fasting state the volume exhaled decreased post-prandially for both the experimental and control groups (34.0 ml kg^{-1} and 20.4 ml kg^{-1} , respectively), and exhalation time was 0.4 s less in both groups. Compared with the fasting

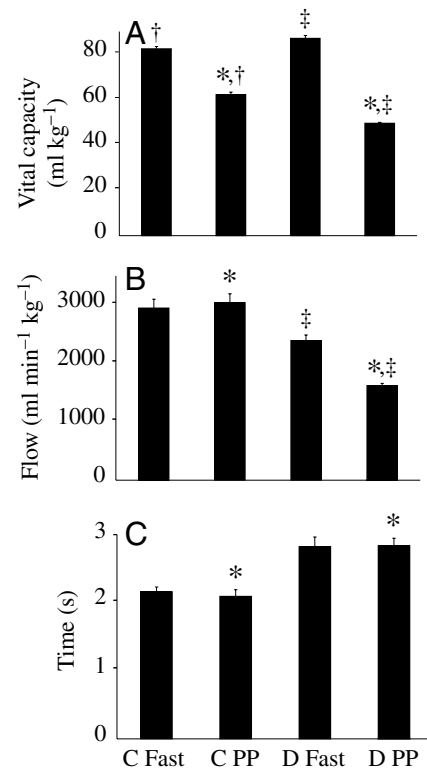


Fig. 3. Study 1. Components of the maximum inhalation that occurred when the animals inspired a 5% hypercapnic anoxic gas over a 3-min period. (A) Relative vital capacity (mean \pm s.e.m.). (B) Relative maximum flow (mean \pm s.e.m.). (C) Duration of inhalation (mean \pm s.e.m.). C Fast, fasting control alligators; C PP, post-prandial control alligators; D Fast and D PP, alligators that had the diaphragmaticus muscle transected and were in the fasting and post-prandial state, respectively. [†]Significant difference ($P \leq 0.05$; t -test) between fasting and post-prandial states within the control group ($N=5$); [‡]significant difference ($P \leq 0.05$; t -test) between fasting and post-prandial states within the experimental group ($N=5$); *significant difference ($P \leq 0.05$; t -test) between experimental and control alligators during post-prandial treatment.

state the maximum flow reached during inhalation decreased post-prandially by $770.7 \text{ ml min}^{-1} \text{ kg}^{-1}$ for the experimental group but there was no change in the control group. Fig. 3 and Table 1 provide a summary of all ventilatory values analyzed in this study.

Study 2: comparison between experimental and control groups

One week, 2 weeks, and 2 months after the surgery no difference in vital capacity was found between the sham and experimental groups. However, 6 months after the surgeries a difference between these groups became apparent in the post-prandial condition, and yielded results in accordance with the data of study 1. Six months after the surgery, in the fasted state, there was once again no significant difference in vital capacity between the experimental and sham groups of animals. However, in the post-prandial state a difference in ventilation

Table 1. Summary of values from Study 1

	C Fast	C PP	D Fast	D PP
VC (ml kg ⁻¹)	82.4±1.0	62.2±1.1	87.0±1.3	49.6±0.4
V _{TE} (ml kg ⁻¹)	78.6±1.5	58.2±1.8	81.0±1.7	47.0±1.4
T _I (s)	2.2±0.07	2.1±0.10	2.8±0.14	2.8±0.11
T _E (s)	1.8±0.10	1.4±0.09	2.1±0.05	1.7±0.06
T _{TOT} (s)	4.0±0.17	3.5±0.16	5.0±0.08	4.5±0.16
Max. inspiratory flow (ml min ⁻¹ kg ⁻¹)	2947.9±152.3	3042.2±149.8	2390.7±97.6	1620.0±38.6
Max. expiratory flow (ml min ⁻¹ kg ⁻¹)	3280.9±169.0	3286.0±142.8	2795.1±57.7	2595.1±169.0

Values are means ± s.e.m., N=5.

C Fast, fasting control alligators; C PP, post-prandial control alligators; D Fast and D PP, alligators that had the diaphragmaticus muscle transected, and were in the fasting and post-prandial state, respectively.

VC, vital capacity; V_{TE}, expiratory volume; T_I, inspiratory duration; T_E, expiratory duration; T_{TOT}, total breath duration.

between the two groups was observed. The vital capacity in the group that underwent transection was significantly less than the vital capacity in the sham group (mean 53.5 ml kg⁻¹ and 71.2 ml kg⁻¹, respectively; $P < 0.05$ *t*-test; Fig. 4). The post-prandial drop in the maximum inspiration, averaged 12% and 28% for sham and experimental groups, respectively.

Study 2: comparison between fed and fasted states

Six months after the surgery, the vital capacity decreased significantly from the fasting state to the post-prandial state for the experimental group and was close to being significant ($P = 0.06$) in the sham group (Fig. 4). The experimental group went from 75.1 ml kg⁻¹ when fasted to 53.5 ml kg⁻¹ when fed,

or a drop in vital capacity equal to 21.6 ml kg⁻¹. Whereas, the sham group went from 81 ml kg⁻¹ when fasted to 71.2 ml kg⁻¹ when fed, or a drop in vital capacity equal to 9.8 ml kg⁻¹. Furthermore, the vital capacity decreased significantly for the average of all the experiments performed (1 week, 2 weeks, 2 months and 6 months after the surgery) in both groups when post-prandial. This represents a drop in volume of 16.7 ml kg⁻¹ for the sham group and a 25.1 ml kg⁻¹ for the experimental group.

Discussion

The most striking results from this study are the following: (1) animals with a transected diaphragmaticus were able to maintain vital capacity in the fasted state, yet lost significantly more volume in the post-prandial state when compared to control animals, and (2) in the post-prandial state both control and experimental animals experienced a very significant decrease in vital capacity. In study 1, a large meal (15% of body mass) decreased the vital capacity by 25% and 43% of the fasting values in animals with intact and transected diaphragmatic muscles, respectively. This represents an 18% difference between the two groups or a 16 ml kg⁻¹ loss in vital capacity for the transected group.

There are several possible explanations for the lack of any effect of transection on vital capacity in fasting animals. It is possible that the diaphragmaticus normally makes negligible contribution to inspiration in fasting, standing animals. If this is the case, it raises the interesting question of whether the same would be true during more taxing times, such as while swimming or running, and there is some evidence that transection of the diaphragmaticus reduces tidal volume during treadmill-exercise in fasting alligators (Hartzler et al., 2004). Alternatively, it is possible that over the course of the year between when the animals underwent surgery and when the experiments were done, the animals gained the ability to compensate for the loss of the diaphragmaticus in the resting state, perhaps by increasing the capacity of other components of their ventilatory mechanism, or by regaining partial use of the diaphragmaticus due to the growth of scar tissue that mechanically reconnected the transected muscle. Although the

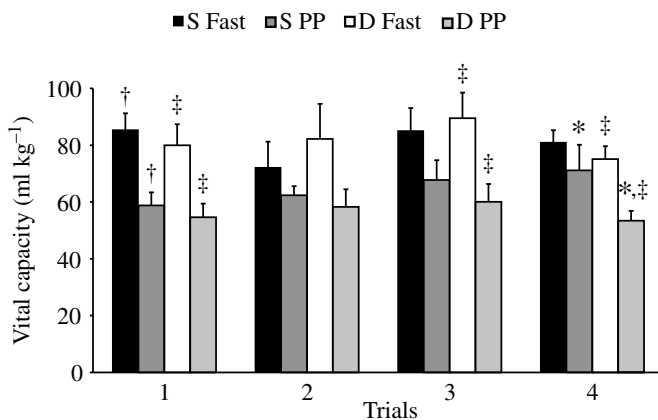


Fig. 4. Study 2. The relative vital capacity (mean ± s.e.m.) of animals breathing a 5% hypercapnic-anoxic or normoxia gas for a 3 min period at 1 week, 2 weeks, 2 months and 6 months after the surgery (trials 1, 2, 3 and 4, respectively). S Fast, fasting sham operated alligators; S PP, post-prandial sham operated alligators; D Fast and D PP, alligators that had the diaphragmaticus muscle transected, and were in the fasting and post-prandial state, respectively. †Significant difference ($P \leq 0.05$, *t*-test) between fasting and post-prandial states within the sham group ($N = 3$); ‡significant difference ($P \leq 0.05$; *t*-test) between fasting and post-prandial states within the experimental group ($N = 3$); *significant difference between experimental and control alligators ($P \leq 0.05$, *t*-test) during post-prandial treatment.

surgery may have eliminated or reduced the ability of the animals to use the hepatic piston for inspiration, costal and pelvic aspiration may have compensated for this loss. Our visual observations of the animals suggest different breathing mechanics are being used.

In order to address the possibility that over time the muscle may have grown back and this was responsible for the results obtained in study 1, or that these results were due to the type of control used (no surgery), or due to the type of gas used (5% CO₂, balance N₂) a second study was conducted. For this study, sham and experimental animals were used to look at the changes in the maximum inspiration 1 week, 2 weeks, 2 months, and 6 months after surgery and with both a hypercapnic–anoxic gas mixture and a hypercapnic–normoxic gas mixture. At 1 week, 2 weeks, 2 months after surgery, the vital capacity obtained were variable and no significant difference was measured. These initial results could be attributed to healing from the surgery, which interfered with the animals' ability to breathe. After 6 months, the results from study 2 matched those of the first study (study 1) remarkably well, in that there was a decrease in the post-prandial vital capacity for both the experimental and control groups (Fig. 4). Six months after the surgery a large meal significantly decreased the maximum volume of inspiration in animals with a transected diaphragmatic muscle when compared to sham operated animals by 21% and 12%, respectively. This represents a 16% difference between the two groups or a 17.7 ml kg⁻¹ greater loss in vital capacity for the transected group. This was a slightly smaller loss in maximum inspiratory volume than was seen in study 1 (18% was found in the first study). This could be due in part to a change in the experimental design where the animals were force fed a homogenized meal that was slightly smaller than the average meal size that the animals chose to eat on their own. Despite this change in protocol the overall trends observed in the main study were again seen for vital capacity. Thus, we think the preponderance of the data indicate that the diaphragmatic muscle makes an important contribution to vital capacity in the post-prandial state.

The observation that the large meal voluntarily eaten by these animals significantly reduced vital capacity in both the control and the experimental groups has some interesting implications for the life history of these animals. Juvenile crocodilians have very high rates of mortality. For example, several studies on both American alligators and on saltwater crocodiles indicate that less than 50% of hatchlings survive to the age of one year [Woodward et al. (Woodward et al., 1987) and references therein]. Because juvenile animals take refuge from predators such as birds and raccoons by submergence,

their abilities to remain submerged may be directly related to their chances of surviving. A decrease in vital capacity may reduce pulmonary oxygen stores and presumably reduce aerobic dive-time. Furthermore, this reduction in lung oxygen stores may occur at a critical time, during the post-prandial period, when metabolic rate and thus the demand for oxygen is known to increase by about fourfold (Busk et al., 2000). An increase in demand for oxygen, concomitant with a decrease in supply, could have significant ramifications. For example, if a juvenile is able to stay submerged aerobically for 6 min in the fasting state, then with a fourfold increase in metabolism during the post-prandial period this would be reduced to about 90 s. A reduction in lung oxygen stores by 43% presumably would reduce this time to about 50 s, a very short time for an animal seeking protection by submergence. Thus an extension of dive-time may be a selective advantage that accompanied the ability of the diaphragmaticus to increase vital capacity during the post-prandial period.

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