SHUNTING AND SURVIVAL IN ALLIGATORS

by

MANUELA NATASCHA GARDNER

B.Sc., The University of Texas at Arlington, 1994

M.Sc., The University of Texas at Arlington, 1996

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Zoology)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September 2004

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ABSTRACT

In terms of cardiac anatomy, crocodilians occupy an intermediate position between the avian and reptilian circulations. They have a completely divided heart but have retained the ability to shunt deoxygenated right ventricular (RV) blood to the body via the left aorta (LAo), which arises from the right ventricle. The LAo continues posteriorly and supplies the digestive system with right ventricular blood during shunting. The central hypothesis of my study was that the ability to shunt RV blood to the digestive system via the LAo aids in the secretion of gastric acid during digestion and/or in reducing the alkaline tide following feeding in alligators (*Alligator mississippiensis*). I used two groups of alligators to investigate the role of the ability to shunt RV blood to the digestive system. Alligators in one group had their LAo surgically tied off and cut (LAo cut; unable to shunt via the LAo) and alligators in the other group had sham surgeries performed on them (LAo intact). I measured growth rates, food intake, water intake, digestion rates, conversion rates, metabolic rates and acid-base status during feeding. Metabolic rates, mass changes, blood variables and acid-base status were also measured during long-term fasting. In addition, I studied the effect of changing body temperature on acid-base status in the two groups of alligators. During fasting, mass changes, blood variables, oxygen consumption ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$), respiratory exchange ratios (RERs) and acid-base status of the two groups of alligators were not significantly different. During feeding, growth rates, digestion rates and conversion rates were similar in the two groups, as were $\dot{V}O_2$ values. Average $\dot{V}CO_2$ and RER values, however, were significantly higher in alligators with

their LAo cut. Changes in acid-base status after feeding were similar between the two groups of alligators. However, when temperature was decreased from 30° C to 20° C alligators with their LAo cut exhibited larger acid-base disturbances during feeding, with significantly higher average pH values. Also, differences observed in acid-base status were reduced or disappeared when feeding was stopped. In summary, the ability to shunt RV blood to the gut after feeding did not benefit digestion or aid in ameliorating the alkaline tide. However, the acid-base status was significantly affected in alligators unable to shunt RV blood to the digestive system via the LAo after feeding and when body temperature was decreased. Thus, during times of additional physiological challenges such as temperature changes, acid-base regulation may become compromised in alligators unable to shunt RV blood to the body. Even so, ligating the LAo did not affect survival in laboratory reared alligators. If shunting RV blood to the body benefits alligators in a natural setting, its role has yet to be determined.

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ACKNOWLEDGMENTS

First, I must thank Dave Jones for his support, kindness, sense of humor and for giving me the opportunity to work with such amazing animals. Dave has been a good advisor and mentor and I am grateful for all his guidance and wisdom. I also thank my committee members Bill Milsom (who was always ready to help and answer questions), Peter Hochachka, Robin Liley and Dave Randall (for his kindness and encouragement).

Special thanks goes to the Jones' lab – Amanda Southwood, Kim Borg, Nicole Elliott and T. Todd Jones for their friendship and help. I would also like to thank Doug Syme for his help when I first arrived in the lab and for his friendship.

I would like to thank all the people of the Zoology department staff, especially the animal care staff at South Campus for their help over the years. Bruce Gillespie, Don Brandys and Granville Williams were invaluable over the years. Allison Barnes has been so helpful and kind.

Many thanks to Jack Herbert for his help on blood sampling in alligators. Also, to Peter Hochachka for letting me use his equipment to measure plasma amino acids. Amanda Southwood, Kim Borg and Michael Pearson helped me wrestle alligators and feed my animals when I was away. Stephane Lair provided great veterinary advice and helped to ensure healthy animals.

I am grateful to Michael Whitlock for taking the time to help me with my data analysis. I am grateful to Agnes Lacombe for her friendship and encouragement. I also thank Amanda Southwood for her great editorial skills.

This thesis would have not been possible without the love and support of my friends and family, especially my sister Mona. Special thanks to Gus, an amazing alligator.

CHAPTER 1

General Introduction

Reptilian cardiac anatomy and physiology

In mammals and birds the mixing of oxygenated and deoxygenated blood within the heart is usually considered a pathological feature. However, in reptiles, the ability to mix oxygenated and deoxygenated blood in the heart, or to redirect oxygenated or deoxygenated blood towards or away from the lungs or body circulation (shunting blood) can be part of their daily routine and may play a vital role in their survival. Among reptiles the mechanisms used to shunt blood varies due to differences in cardiac and central circulatory anatomy, which have been reviewed in several papers (White, 1968; Webb *et al.*, 1971; Webb *et al.*, 1974; Van Mierop and Kutsche, 1985).

All reptiles have a complete atrial septum. Among reptiles, the ventricle in chelonians and squamates is subdivided into three interconnected cava (Figure 1.1). Generally, a thick muscular septum (Muskelleiste or horizontal septum) divides the ventricle into a smaller cavum pulmonale (CP) and a larger cavum dorsale, which is further divided into cavum arteriosum (CA) and cavum venosum (CV) by a vertical septum. Three vessels arise from the ventricle. The pulmonary trunk originates from the CP and the two aortic arches (right and left aorta; RAo and LAo, respectively) arise from the CV, with the LAo adjacent to the CP. This cardiac anatomy allows for intracardiac mixing of oxygenated and deoxygenated blood rather than separation.

In fact, two types of shunts have frequently been described in non-crocodilian reptiles. A right to left shunt ($R \rightarrow L$) represents a fraction of de-oxygenated systemic venous blood being recirculated to the body, bypassing the lungs. A left to right shunt ($L \rightarrow R$) represents a fraction of oxygenated pulmonary venous blood flowing back into the pulmonary circulation. In non-crocodilian reptiles, the anatomical arrangement of the cava allows for bidirectional shunting, i.e. $R \rightarrow L$ and $L \rightarrow R$ shunting can occur simultaneously (Heisler and Glass, 1985; Hicks and Comeau, 1994). Consequently, shunting in these animals must be discussed in terms of whether the $R \rightarrow L$ shunt or $L \rightarrow R$ shunt predominates.

The washout and the pressure hypotheses have been proposed as mechanisms of intracardiac shunting in non-crocodilian reptiles (White and Ross, 1966; Shelton and Burggren, 1976; Heisler *et al.*, 1983; Heisler and Glass, 1985; Hicks and Malvin, 1995; Hicks *et al.*, 1996). The basic feature of the washout hypothesis is that the CP is separated from the CV and the CA during systole by the horizontal septum (Figure 1.2). During diastole, the CA receives blood from the left atrium (oxygenated blood) and the CP receives blood from the right atrium through the CV (deoxygenated blood) and any blood remaining from the previous systole in the CV (i.e. end-systolic volume) is washed into the CP by venous blood returning from the body. During systole, blood from the CA is ejected through the CV into the right and left aortic arches (RAo and LAo) along with any deoxygenated blood that remained within the CV during diastole. Blood from the CP is ejected into the pulmonary artery. The magnitude of a $R \rightarrow L$ shunt depends on the volume of systemic venous blood within the CV at the end of diastole

which is blood that normally would be sent to the lungs. The volume of blood within the CV at the end of systole is of pulmonary venous origin (oxygenated blood) and this determines the magnitude of the L→R shunt. This blood is washed into the CP during diastole and consequently sent to the lungs.

The general feature of the pressure hypothesis is that the horizontal septum does not completely separate the CP from the CV and CA. Here, the magnitude of shunting depends on the vascular resistances of the outflow tracts. When pulmonary resistance is high, relative to systemic resistance, blood from the CP is ejected around the horizontal septum into the aortic arches (R→L shunt) with the majority of shunted blood being directed into the LAo due to its anatomical position (Figure 1.1; White and Ross, 1966; Shelton and Burggren, 1976; Heisler and Glass, 1985; Hicks and Malvin, 1995). When pulmonary resistance is low, a fraction of blood from the CV and CA is ejected around the horizontal septum into the CP and thus the pulmonary artery. This is a L→R shunt.

Non-crocodilian reptiles may exhibit either the washout or pressure mechanism during shunting, and both mechanisms may be displayed within a given species. In varanids the atrio-ventricular valves are large enough to prevent mixing of oxygenated and deoxygenated blood during diastole and the CP and CV are completely separated by the horizontal septum during systole (White, 1959; White, 1968; Webb *et al.*, 1971; Heisler *et al.*, 1983; Heisler and Glass, 1985; Hicks *et al.*, 1996). In these animals intra-cardiac shunting appears to be mainly due to the washout mechanism (Heisler *et al.*, 1983; Ishimatsu *et al.*, 1988). Blood remaining in the CV at the end of systole (oxygenated blood) is washed into the CP during the next diastole and blood at the end of

diastole (deoxygenated blood) in the CV is ejected into the aortic arches during the following systole. Ishimatsu *et al.* (1988) found that during a R→L shunt in *Varanus niloticus* most of the deoxygenated blood from the CV was ejected into the LAo and that RAo blood represented primarily left atrial blood i.e. oxygenated blood. This is due to the anatomical arrangement of the chambers and aortic arches of the varanid heart. During systole, most of the deoxygenated blood remaining within the CV is ejected into the LAo due to its more caudal position (Figure 1.2). The RAo lies closer to the CA and during systole primarily receives oxygenated blood. A more recent study (Wang *et al.*, 2002) showed that pythons (*Python molurus*) also exhibit a large degree of flow separation between pulmonary and systemic circulation as has been found in varanid lizards. This is thought to be due to a well developed horizontal septum and a small CV in Pythons (Webb, 1979; Farrell *et al.*, 1998).

In turtles the volume of the CV is larger than in varanids. Also, the potential for intracardiac mixing of oxygenated and deoxygenated blood occurs during diastole due to the incomplete separation of the chambers by the atrio-ventricular valves (Hicks and Malvin, 1992; Hicks and Comeau, 1994). In the red-eared pond slider turtle (*Pseudemys scripta*), R→L shunting is due to the washout mechanism (Hicks and Malvin, 1992; Hicks and Comeau, 1994). However, Hicks *et al.* (1996) showed that a net L→R shunt in *Pseudemys scripta* is due to both washout and pressure mechanisms. During early systole, when systemic resistance was higher than pulmonary, the pulmonary valves open before the aortic valves and blood from the CV is pushed around the horizontal septum into the pulmonary circulation and even blood from the CA may reach the CP. On the

other hand, oxygenated blood will now be in the CV, reducing the magnitude of the R→L washout shunt. During the later phase of systole the cava are completely separated by the horizontal septum, so, in contrast to the situation observed in varanids, the shunts are set up both in diastole and early systole in the turtle.

Intracardiac shunting in non-crocodilian reptiles can have pronounced consequences by affecting oxygen (O_2) and carbon dioxide (CO_2) levels in the systemic and pulmonary circulations. Generally, a R→L shunt lowers the arterial oxygen content in the systemic circulation relative to that entering the heart from the lungs and a L→R shunt raises the oxygen content in the blood going to the lungs. The oxygen dissociation curve (ODC), which describes the relationship between the PO_2 and the oxygen saturation, has become an important tool in understanding the effect of R→L shunting on arterial and mixed venous oxygen contents (Figure 1.3). When a R→L shunt is present, systemic arterial PO_2 , O_2 content and HbO_2 saturation are all decreased relative to pulmonary venous blood returning from the lungs. However, the O_2 and CO_2 content of arterial blood and mixed venous blood depend on many additional factors such as inspired PO_2 , O_2 carrying capacity of the blood, breathing pattern, pulmonary and systemic blood flows and O_2 extraction rates by the tissues.

Figure 1.3. Effects of a R→L shunt on systemic arterial O_2 content as demonstrated by the O_2 dissociation curve. $[O_2]$ = Oxygen content; C_{pv} = pulmonary venous oxygen content; Ca = arterial oxygen content; C_v = mixed venous oxygen content. The arrow indicates the lower arterial oxygen content and lower mixed venous oxygen content when a R→L shunt is present. Adapted after Hicks and Wang, 1996.

Arterial oxygen content (Ca) is determined by the shunt fraction (Q_s/Q_T where Q_s is the shunt flow and Q_T is the total blood flow to the systemic circulation), the non-shunt fraction (Q_{ns}/Q_T where Q_{ns} is the blood flow from the lungs) and the O_2 contents of the mixed venous (C_{vO_2}) and lung (C_{pO_2}) blood (e.g. Rossoff *et al.*, 1980; Wood, 1982, 1984). Figure 1.3 shows how the presence of a R→L shunt reduces arterial oxygen content. When no R→L shunt is present Ca is equal to C_{pv} , but in the presence of a R→L shunt Ca is reduced depending on the aforementioned factors and consequently C_v will be reduced as well.

Rossoff *et al.* (1980) described how a right shift of the ODC (i.e. the Bohr shift indicative of decreasing oxygen affinity) would be beneficial during the presence of a R→L shunt as long as inspired PO_2 is normoxic and blood is equilibrating to full saturation (lung PO_2 lies on the flat portion of the ODC). A right shift of the ODC would increase arterial PO_2 as well as mixed venous PO_2 during environmental normoxia (Figure 1.4). In animals with R→L shunts, shifting the ODC to the right and thus

decreasing O_2 affinity of the blood would enhance O_2 delivery to the tissues when demand is high, i.e. during an increase in body temperature or during exercise. Without a concurrent right shift of the ODC, a R→L shunt would decrease arterial PO_2 levels even if ventilation is matched to the increase in oxygen demand. Wang and Hicks (1996b) showed that when lung PO_2 is high in turtles, an increase in lung ventilation would not increase O_2 content of the blood leaving the lung because it would be already 100% saturated. Yet, the presence of a R→L shunt will reduce arterial PO_2 levels. Also, Wood (1982) found that increased body temperatures in *Varanus exanthematicus* resulted in a significant increase in arterial PO_2 . This increase in arterial PO_2 could have been due to a right shift of the ODC via temperature effects, a decrease in R→L shunt, an increase in systemic blood flow and thus increase in mixed venous O_2 content, an increase in left atrial oxygen content or a combination of the aforementioned.

Figure 1.4. A simple graphical representation on how a right shift of the ODC would be beneficial during the presence of a R→L shunt. $\%O_2$ = Oxygen saturation; C_{pv} = pulmonary venous oxygen content; S_a = arterial oxygen saturation; C_v = mixed venous oxygen saturation. The arrow indicates the right shift of the ODC. Adapted from Rossoff *et al.*, 1980).

Reptiles are intermittent breathers and so lung PO_2 is much more variable compared with animals that continuously ventilate. Reptiles also experience large variations in body temperatures, which will result in shifts of the ODC. For example,

lizards have high preferred body temperatures (35-40° C) and low O₂ affinity blood (Pough, 1980). Wood (1984) pointed out that when external hypoxia (low inspired PO₂) is combined with that of internal hypoxia (such as a R→L shunt), a decrease in O₂ affinity will only be beneficial to a point which varies with shunt fraction, inspired PO₂, lung PCO₂ and the respiratory properties of the blood. If the shunt is small, arterial O₂ content will be on the flat portion of the curve and thus a right shift will not significantly affect arterial PO₂. Increasing the shunt fraction will place the arterial O₂ content on the steep portion and now a right shift of the curve will increase arterial PO₂.

A R→L shunt also affects the CO₂ dissociation curve due to the Haldane effect as well as CO₂ levels in the arterial blood. A reduction in systemic arterial oxygenation will shift the CO₂ dissociation curve to the left and upward. The systemic PCO₂ depends on the amount of venous admixture and so arterial PCO₂ could be lower, higher or the same as pulmonary venous PCO₂. This in turn could have consequences concerning arterial pH. An increase in arterial PCO₂ would decrease pH and this will affect oxygen affinity (right shift of ODC).

Thus, the advantages of the ability to shunt are not intrinsically obvious and many questions remain regarding the utility and purpose of the shunt. Under what circumstances do non-crocodilian reptiles shunt? How are the two different shunts regulated? What are the potential benefits of shunting in non-crocodilian reptiles?

Reptiles exhibit an intermittent breathing pattern characterized by ventilatory and non-ventilatory periods and changes in the ventilatory state will affect respiratory variables (O₂, CO₂ and pH) in the whole body. During breath holding, O₂ is extracted

from the lungs at a rate dependant on tissue metabolism and CO_2 is buffered by body stores which are rapidly released when ventilation resumes. As the apnoeic period progresses, lung PO_2 is reduced, pulmonary venous blood will no longer be completely saturated and systemic oxygen delivery is reduced. In non-crocodilian reptiles the absence of a divided heart makes it possible to have independence of flows to the lung and body. These animals can perfuse their lungs at variable flow rates not related to the systemic circulation. During apnoeic periods flow to the lungs is decreased and during ventilation flow to the lungs is increased. Hence, it has been suggested that the presence of shunts may be an adaptation to episodic breathing and the diving habits of these animals (e.g. White, 1978; Wood, 1984; Shelton, 1993).

In turtles, the direction of net shunting seems to be correlated to the ventilatory state of the animals i.e. a net $\text{R} \rightarrow \text{L}$ shunt during apnoea and a net $\text{L} \rightarrow \text{R}$ shunt during breathing. This situation is referred to as cardiorespiratory synchrony (Millen *et al.*, 1964; White and Ross, 1966; Shelton and Burggren, 1976; Burggren and Shelton, 1979; Heisler and Glass, 1985; White *et al.*, 1989; Wang and Hicks, 1996a). For example, in freely diving freshwater turtles (*Trachemys scripta*), the $\text{R} \rightarrow \text{L}$ shunt fraction increased during non-ventilatory periods and the $\text{L} \rightarrow \text{R}$ shunt fraction increased during ventilatory periods (Wang and Hicks, 1996a). On the other hand, Heisler *et al.* (1983) found no relationship between shunting and ventilatory state in *Varamus exanthemicus*. The magnitude of $\text{R} \rightarrow \text{L}$ and $\text{L} \rightarrow \text{R}$ shunting during apnoea or ventilation in varanus were not significantly different.

Several studies have shown that cardiovascular changes during breath holding and the development of a net R→L shunt are under cholinergic control (White, 1976; Milsom *et al.*, 1977; Hicks and Malvin, 1992; Hicks, 1993; Hicks, 1994; Hicks and Comeau, 1994; Hicks *et al.*, 1996; Wang *et al.*, 2001b). The efferent vagus innervates the heart, smooth muscles of the lungs and the smooth muscle in the pulmonary artery of reptiles. Electrical stimulation of the vagus nerve results in an increase in pulmonary vascular resistance in turtles, lizards and snakes, promoting a net R→L shunt. The R→L shunt is abolished by the addition of atropine, which is a competitive antagonist of muscarinic cholinergic receptors and blocks cholinergic vasoconstriction of the pulmonary circulation (Berger, 1972; Burggren, 1977; Milsom *et al.*, 1977; Smith and McIntyre, 1979; Donald *et al.*, 1990; Comeau and Hicks, 1994; Hicks and Comeau, 1994). In contrast, the L→R shunt is adrenergically mediated.

Hicks and Malvin (1992) showed that a net R→L shunt was induced via vagal stimulation and acetylcholine (ACH) administration in *Pseudemys scripta* and this was evident in arterial blood gas measurements. The partial pressure of oxygen (PO₂) in the RAo was 85 mmHg and 75 mmHg in the LAo prior to ACH administration. The PO₂ fell to 50 mmHg in the RAo and 40 mmHg in the LAo after ACH administration, indicating a net R→L shunt. Similar changes in blood PO₂ were also found during voluntary diving in turtles and it was concluded that the changes were due to a R→L shunt induced by cholinergic mechanisms (White *et al.*, 1989). This was confirmed in *Pseudemys scripta* by right vagal efferent stimulation which produced a 57% reduction in heart rate, 71% reduction in pulmonary blood flow, a 222% increase in pulmonary

vascular resistance and a significant decrease in PO_2 in the LAo (Hicks, 1993).

Interestingly, RAo PO_2 was not significantly affected during right vagal efferent stimulation, suggesting differential distribution of blood into the aortic arches as has been found in varanids (Ishimatsu *et al.*, 1988). Most of the blood within the RAo represented left atrial blood and blood within the LAo was primarily deoxygenated blood from the CV.

In *Pseudemys scripta* L→R shunting was induced by vagal afferent stimulation (Hicks *et al.*, 1996). Injection of epinephrine resulted in an increase in pulmonary blood flow and a reduction in pulmonary vascular resistance and this response was diminished by adrenergic blockage (Lillywhite and Donald, 1989; Comeau and Hicks, 1994; Overgaard *et al.*, 2002). Thus, R→L shunting is cholinergically mediated and L→R shunting is adrenergically mediated in non-crocodilian reptiles.

It has been proposed that a R→L shunt may aid in O_2 uptake from the lungs, to sequester CO_2 away from the lungs or to enhance tissue CO_2 storage during diving (White, 1985; Grigg and Johansen, 1987; Hicks and Wang, 1996). In non-crocodilian reptiles, a R→L shunt reduces flow to the lungs, arterial O_2 content and venous O_2 content during a dive if O_2 uptake by the tissues remains the same. So, venous blood returning to the lungs should take up more O_2 due to the bigger concentration gradient (pulmonary oxygen uptake: $Q_p * (\text{arterial} - \text{venous}) O_2 \text{ difference } (CaO_2 - CvO_2)$ across the lung; where Q_p – pulmonary blood flow). Thus, O_2 uptake remains constant even with the reduced pulmonary blood flow due to the R→L shunt. In the lungs, the blood oxygen capacitance coefficient (β) is increased due to the R→L shunt and this offsets the

reduction in pulmonary blood flow. This strategy would not prolong the ability to dive longer if tissue O₂ uptake remained the same. In turtles, transient increases in pulmonary flow have been observed causing brief reductions in the R→L shunt which allows metering of O₂ stores (Burggren and Shelton, 1979). Lung perfusion is briefly increased during a dive and the R→L shunt briefly reduced for the purpose of O₂ uptake. During lung perfusion, the blood O₂ stores are replenished and the lung O₂ stores are reduced. Doing this several times during apnoea would not prolong the apnoeic period as the O₂ stores of both the blood and the lungs are decreased at a rate equal to tissue metabolic rate if O₂ demands remain the same.

A reduction in oxygen delivery could induce a hypometabolic state (Jackson, 1968; Hochachka *et al.*, 1996; Hicks and Wang, 1999), which may involve changes in membrane permeability, reductions in active membrane transport and reductions in protein synthesis (Buck and Hochachka, 1993; Land *et al.*, 1993; Hochachka *et al.*, 1996, 1997). For example, Buck and Hochachka (1993) showed that protein synthesis fell by 92% during anoxia in hepatocytes from the turtle *Chrysemys picta bellii*. A R→L shunt lowers arterial PO₂ levels and thus could be used as a strategy to induce a hypometabolic state of tissues during a dive. This idea was tested in freshwater turtles by Hicks and Wang (1999). They showed that hypoxia produced a decrease in O₂ uptake in anesthetized turtles. Also, the arterial O₂ levels measured in their anaesthetized turtles were similar to values measured in conscious turtles after the development of a R-L shunt during a dive (Burggren and Shelton, 1979; White *et al.*, 1989). Oxygen uptake increased after the injection of 2, 4-dinitrophenol, which uncouples oxidative

phosphorylation from electron transport by making the mitochondrial cristae membrane more permeable to hydrogen ions, suggesting that the hypometabolic state was due to a decrease in ATP demand. A more recent study by Platzack and Hicks (2001) showed that vagal efferent stimulation resulted in a decrease in O₂ uptake and a decrease in arterial PO₂ values in anesthetized turtles and confirmed that injection of 2, 4-dinitrophenol increased O₂ uptake. They attributed the decrease in O₂ uptake to an overall decrease in tissue metabolic rate, i.e. a hypometabolic state. Earlier studies showed that right vagal efferent stimulation resulted in a net R-L shunt in turtles and in reductions of PO₂ values in the systemic circulation (Hicks and Malvin, 1992; Hicks, 1993; Hicks and Comeau, 1994). Thus, a R→L shunt may be a strategy used in turtles during a dive to induce a hypometabolic state and thus prolong the dive. An increase in CO₂ content may also act as a metabolic depressant. Harken (1976) showed that O₂ consumption rates decreased with an increase in arterial PCO₂ in the isolated canine hindlimb.

In varanid lizards and *Python molurus*, pressure separation of the pulmonary and systemic circulations has been demonstrated (Burggren and Johansen, 1982; Heisler *et al.*, 1983; Wang *et al.*, 2002). During times of increased metabolic demands, such as activity and/or digestion, reducing the net R-L shunt fraction and so increasing arterial PO₂ would ensure adequate O₂ delivery to the active tissues (Wang *et al.*, 2001a). For example, Gleeson *et al.* (1980) showed that *Varanus exanthematicus* had a high arterial PO₂ (94 mmHg at 35° C) and an increased arterial-venous O₂ difference (due to increased O₂ extraction by the tissues) during exercise. The increased arterial-venous O₂ difference

promoted O₂ uptake at the lungs. In snakes, large increases in metabolic rate following feeding have been observed (Secor and Diamond, 1995). Again, oxygen delivery to the tissues is important during that time and it is thought that reducing or abolishing the R→L shunt would be beneficial in ensuring adequate oxygen delivery.

Another hypothesis is that R→L shunting reduces CO₂ transfer into lungs (White, 1978; 1985) so that metabolically produced CO₂ is not stored in the lungs during a breath hold but is recirculated to the body. This results in a lower respiratory exchange ratio (RER; $\dot{V}CO_2/\dot{V}O_2$) across the lungs (Ackerman and White, 1979; Burggren and Shelton, 1979; Hicks and White, 1992). For example, in alligators O₂ is extracted from the lungs at a constant rate during breath holding, but CO₂ is buffered by body stores (Hicks and White, 1992). This results in RER values of < 0.7. An increase in tissue CO₂ content would favour O₂ delivery to the tissues i.e. right shifting the ODC (Bohr effect). Paradoxically, increasing O₂ delivery to the tissues via CO₂ retention would diminish O₂ stores more rapidly and thus reduce the time of breath holding.

Burggren (1982) proposed that a R→L shunt may aid in the prevention of a “wet lung”. When pressure inside capillaries is high, filtration of fluid from the plasma into the interstitial space takes place. Filtration of the fluid depends on the difference between the blood pressure and the colloidal osmotic pressure and the permeability of the capillary wall. Burggren (1982) suggested that in the lungs of turtles, plasma filtration increases with an increase in pulmonary flow during ventilation and decreases when pulmonary perfusion is low during breath holding so that plasma is reabsorbed. Thus, reducing the flow to the lungs during breath holding via a R→L shunt would promote

plasma reabsorption and prevent fluid build up in the lung. In *Pseudemys scripta* net reabsorption of fluid was found during apnoea and net plasma filtration during lung ventilation (Burggren, 1982). Burggren did point out that the extent of a wet lung in the turtle would depend on the effectiveness of the lymphatic system. Normally, any accumulated fluid is drained by the lymphatic system. For example, in the amphibian *Bufo marinus*, the lymphatic system was efficient enough to remove any filtered plasma (Smits, 1994). Whether or not this is true in reptiles has not been determined and there have been no further experiments to investigate this hypothesis.

A net L→R shunt may facilitate carbon dioxide (CO₂) removal after a breath hold (Ackerman and White, 1979). RER values above 1 have been found in ventilating reptiles (Burggren and Shelton, 1979; Hicks and White, 1992). During breath holding, CO₂ accumulates in the tissues and blood and hyperperfusing the lungs following a breath hold may aid in the removal of accumulated CO₂. This is based on the phenomenon of the Haldane effect, which refers to the greater ability of reduced Hb to form carbaminohemoglobin, thereby buffering H⁺. The Haldane effect decreases blood CO₂ capacitance at the lung and so enhances CO₂ removal. In non-crocodilian reptiles, oxygenated blood returning from the lungs is mixed with deoxygenated blood returning from the tissues during a L→R shunt. This increases the HbO₂ saturation of the pulmonary arterial blood relative to right atrial blood and thus pulmonary arterial PCO₂. The higher PCO₂ gradient across the lungs facilitates CO₂ excretion. This phenomenon has not been thoroughly investigated in reptiles. It should be noted CO₂ can be rapidly

blown off by hyperventilation. Also, crocodilians, which do not exhibit a L→R shunt, can effectively remove excess CO₂.

Another hypothesis regarding L→R shunts is that it may aid in ventilation and perfusion matching ($\dot{V}/\dot{Q}$) during ventilation (West *et al.*, 1992) via an increase in lung perfusion during ventilation. This would reduce physiological shunting due to $\dot{V}/\dot{Q}$ mismatching. However, studies have shown that an increase in pulmonary blood flow does not significantly increase the PO₂ of pulmonary venous blood (Hicks and Malvin, 1992; Hicks and Comeau, 1994). Also, a L→R shunt may negatively affect O₂ exchange, as the increase in PO₂ of pulmonary arterial blood will decrease the O₂ gradient across the lungs, resulting in decreased O₂ uptake which would be unfavourable. Also, increasing cardiac output alone increases pulmonary blood flow, so a L→R shunt is not required to ensure $\dot{V}/\dot{Q}$ matching.

Shunting may also play a role in thermoregulation in reptiles (Burggren, 1987). A net R→L shunt may facilitate warming by perfusing the periphery with relatively cooler blood during basking. Baker and White (1970) found that during warming a net R→L shunt was exhibited in lizards as well as an increase in heart rate and cardiac output. On the other hand, a L→R shunt would facilitate cooling by recirculating warmer blood through the lungs (Tucker, 1966; Crawford and Gatz, 1974).

Burggren (1987) suggested that a R→L shunt may help to save cardiac energy by reducing pulmonary perfusion during apnoea. However, this energy saving would only be 1% of total metabolic rate in a turtle and may not significantly affect daily energy expenditure.

It is apparent from the above studies that the physiological role (if any) of shunting in non-crocodilian reptiles has not been clearly determined. Because of the variation of the ventricular anatomy among non-crocodilian reptiles (Van Mierop and Kutsche, 1985) and the different physiological requirements of these animals, future studies for different physiological states (exercise, digestion, resting, fasting, diving) may reveal a sole physiological role, or perhaps myriad of potential functions for shunting in non-crocodilian reptiles. In a recent review, Hicks (2002) pointed out that the ability to shunt in reptiles may not be a feature that was selected for, rather it simply was not selected against. Even though shunting will affect blood flow and blood gases, these changes may not have negative consequences on survival in reptiles.

For over 170 years comparative physiologists have been excited by the unique anatomy of the crocodilian heart and consequently it has been described on many occasions and in great detail (for example, Panizza, 1833; Reese, 1915; Bolk *et al.*, 1933; White, 1968; Grigg, 1991; Shelton and Jones, 1991; Jones and Shelton, 1993; Jones, 1996; Axelsson and Franklin, 1997). Unlike other reptiles, crocodilians have a complete interventricular septum that divides the heart into two separate chambers (Figure 1.5). The right aorta (RAo) arises from the left ventricle (LV) and the right ventricle (RV) gives rise to two vessels, the pulmonary artery (PA) as well as the left aorta (LAo). Just proximal to the pulmonary valves, the pulmonary outflow tract contains small dense fibrous nodules arranged in a circular manner (cog-wheel valve; Webb, 1979; van Mierop and Kutsche, 1985; Axelsson *et al.*, 1996). There are two sites of communication

between the RAo and LAo. The first site is just outside the heart via the foramen of Panizza, a small opening in the common wall between the RAo and LAo (Panizza, 1833). The second site is an arterial anastomosis which lies posterior to the heart (Figure 1.5). The RAo, after giving rise to the carotid and subclavian arteries, continues posteriorly as the dorsal aorta and supplies the body with oxygenated blood. The LAo continues posteriorly and branches, just before the anastomosis, to perfuse the stomach, small intestine, pancreas, spleen and gall bladder (Figures 1.5 and 1.6). With this unique cardiac anatomy blood may flow between the two aortic arches via the two connections and there is also the potential for shunting right ventricular blood to the digestive system via the LAo (pulmonary to systemic shunt, P→S which is termed a R→L shunt in non-crocodilian reptiles; Jones, 1996).

The circulatory events during non-shunting and shunting periods have been described in detail by Shelton and Jones (1991) and Jones and Shelton (1993), so I will describe them in brief. Ventricular filling occurs during diastole. Also, flow from RAo to LAo occurs through the foramen of Panizza during this time. During early systole oxygenated blood from the LV is ejected into the RAo and blood from the RV into the PA. Pulmonary blood flow starts about 80 milliseconds before flow from the LV into the RAo begins (Shelton and Jones, 1991). This difference in ejection timing is due to the lower pressure in the pulmonary circuit allowing pulmonary valves to open sooner. Blood flow through the anastomosis is from RAo to LAo (Figures 1.5 and 1.6). Flow

within the LAo is towards the heart at this point (around the loop; Jones and Shelton, 1993).

During late systole flow to the lungs is terminated by active closure of the cog-wheel valve, and when no shunt is occurring, the LV continues to eject blood into the RAo and flow within the LAo is still towards the heart due to flow via the anastomosis. During late systole the myocardium of the pulmonary outflow tract contracts and the subpulmonary nodules come tightly together, preventing flow to the lungs. This is apparent from pressure recordings which showed a large rise in pressure within the RV during late systole (Shelton and Jones, 1991; Jones and Shelton, 1993).

During shunting periods, the events during early systole are the same as during non-shunting periods and during late systole; blood from the LV is ejected into the RAo as before. After the active closure of the cog-wheel valve, RV pressure rises, exceeds that of LAo pressure and RV blood is ejected into the LAo.

The cog-wheel valve, foramen of Panizza and arterial anastomosis are important sites for control and blood flow distribution of the crocodilian heart. The pattern of flow through the foramen has been of interest for many years (Fritsch, 1869; Sabatier, 1873; Greenfield and Morrow, 1961; White, 1969; Grigg and Johansen, 1987; Axelsson *et al.*, 1989; Jones and Shelton, 1991; Malvin *et al.*, 1995). For example, White (1969) suggested that flow through the foramen occurred during systole and was from RAo to LAo. However, several studies have shown that the foramen is closed during systole via the medial cusp of the RAo valve and so flow through the foramen was found only during diastole (Axelsson *et al.*, 1989; Shelton and Jones, 1991; Jones and Shelton,

1993). Grigg and Johansen (1987) proposed that the foramen of Panizza is of variable calibre and Karila *et al.* (1995) showed that the foramen and surrounding tissue contain smooth muscle cells, which are richly innervated, and these may function in regulating the diameter of the foramen. A more recent study by Axelsson and Franklin (2001) confirmed that bolus injections of adrenaline, which causes vasoconstriction in smooth muscle, decreased the diameter of the foramen, which was reversed after the addition of the potent vasodilator vasoactive intestinal peptide (VIP). Shelton and Jones (1991) stated that due to the small flow through the foramen, its function may be to prevent clotting of blood within the LAo during times of non-shunting.

The anastomosis is heavily innervated and its diameter can be changed with pharmaceutical substances (Karila *et al.*, 1995; Axelsson and Franklin, 2001; Axelsson *et al.*, 2001). For example, Axelsson *et al.* (2001) found that adrenaline resulted in contraction of the anastomosis and Karila *et al.* (1995) found that substance P and Neuropeptide Y both increased blood flow through the anastomosis.

The cog-wheel valve located in the subpulmonary conus has been of interest in the regulation of pulmonary blood flow and in setting up a P→S shunt in crocodilians (Greenfield and Morrow, 1961; Grigg and Johansen, 1987; Axelsson *et al.*, 1989; Shelton and Jones, 1991; Jones and Shelton, 1993; Axelsson *et al.*, 1997; Franklin and Axelsson, 2000; Syme *et al.*, 2002). The valve is surrounded by cardiac muscle (van Mierop and Kutsche, 1985). Contraction of this muscle causes the closure of the valve, increasing pulmonary outflow resistance and resulting in a secondary rise in RV pressure seen during each cardiac cycle (e.g. Greenfield and Morrow, 1961; Shelton and Jones, 1991;

Jones and Shelton, 1993). The second rise in RV pressure, if large enough will favour the development of a shunt. A study by Syme *et al.* (2002) showed that inhibition of cog-wheel valve contraction prevented the development of a P→S shunt. After the injection of ethanol into muscle surrounding the cog-wheel valve, the secondary rise in RV pressure was abolished and a shunt could not develop.

A P→S shunt can be induced mechanically, by neural stimulation or by pharmaceutical manipulation (Jones and Shelton, 1993; Karila *et al.*, 1995; Malvin *et al.*, 1995; Axelsson *et al.*, 1989; Holmgren *et al.*, 1989; Axelsson *et al.*, 1997; Syme *et al.*, 2002). For example, occlusion of the RAo just anterior to the anastomosis increased RAo pressure and decreased LAo pressure in the saltwater crocodile *Crocodylus porosus* (Axelsson *et al.*, 1997). The decrease in LAo pressure was large enough to result in a P→S shunt. As in non-crocodilian reptiles, vagal stimulation maintained or produced a P→S shunt in alligators (Malvin *et al.*, 1995). Pulmonary resistance increased in *Caiman crocodylus* when ACH was injected into the femoral vein (Axelsson *et al.*, 1989), which leads to the opening of the LAo valve and thus a shunt.

The crocodilian cardiac anatomy favours a P→S shunt (Jones, 1996). The RV in crocodilians has a thick wall and is capable of producing high pressures exceeding LAo pressure. Also, the anastomosis is situated in such a way as to favour flow from LAo to RAo (Figure 1.5). For a P→S shunt to occur, RV pressure has to exceed LAo pressure. This can be achieved by increasing the end-diastolic volume of the RV (i.e. the Starling

effect), by increasing the pulmonary vascular resistance, or by decreasing the systemic vascular resistance by decreasing LAo pressure (Jones and Shelton, 1993; Franklin and Axelsson, 1994; Syme *et al.*, 2002).

A P→S shunt can be induced in a laboratory setting, but when do alligators shunt “naturally”? Early studies showed that alligators developed a P→S shunt during forced submersion (White, 1956) but other studies have shown that freely diving crocodilians do not always develop a P→S shunt (Smith *et al.*, 1974; Grigg and Johansen, 1987). Unpublished laboratory observations show that alligators do not shunt when upset or stressed. Unfortunately, a stressed state is all too easy to induce in alligators. Jones *et al.* (in preparation) found that alligators shunt almost continuously when quiescent i.e. when left alone. Hence, alligators shunted as much as 85% of the time. They also found no relationship between ventilatory state and shunting in alligators in our laboratory (unpublished data). Once the shunt is operative, the alligator continues to shunt regardless of ventilatory state (apnoea or breathing).

Arterial blood flow to the splanchnic region is via the coeliac trunk and the mesenteric arteries. Digestion results in a pronounced postprandial hyperaemia which can be accomplished by an increase in cardiac output, the redistribution of blood flow or both. This is regulated by intrinsic (local metabolic and myogenic factors such as locally produced vasoactive substances) and extrinsic (sympathetic innervation, circulating vasoactive substances) mechanisms (Granger *et al.*, 1980). In crocodilians, the coeliac artery is the continuation of the LAo and Axelsson *et al.* (1997) suggested that an

increase in vascular resistance in the anastomosis (which connects the RAo and LAo posterior to the heart; Figure 1.5 and 1.6) would isolate the coeliac vasculature from the RAo and thus LAo pressure would be reduced. This would favour a P→S shunt and may be of benefit during the postprandial state in crocodilians when coeliac vascular resistance is low.

When first arriving in the laboratory, I determined the regional blood flow distribution during non-shunting and shunting periods in alligators (in preparation). Shunting, which occurred naturally in these alligators and non-shunting periods were determined from blood flow and blood pressure tracings that were monitored continuously in awake alligators and recorded during the experiments. I injected fluorescent microspheres into the RAo and LAo during non-shunting and shunting periods. During non-shunting periods all tissues analyzed were perfused by the RAo and microspheres injected into the LAo were primarily found within the spleen, gallbladder and stomach (Figure 1.7). We used relatively small microspheres (25 μm in diameter) and the spleen contained a large number of microspheres/g tissue when perfused by the LAo due to its anatomical position (Figure 1.8). During shunting periods microspheres injected into the LAo were primarily found in organs associated with digestion such as stomach, small intestine, pancreas and gallbladder. Interestingly, the kidneys also primarily received LAo blood (i.e. RV blood) during shunting periods (Figure 1.7). Also, the brain received most of its blood from the RAo during non-shunting and shunting periods as would be expected because the carotid artery branches off the RAo

(Figure 1.5). Another interesting result was that blood flow distribution from the RAo was different during shunting periods compared with non-shunting periods. The spleen, gall bladder, stomach, kidneys and small intestine received significantly less blood from the RAo during shunting periods. These results suggest that during shunting periods the digestive system is primarily perfused with RV blood. So, why would alligators send RV blood to the digestive system?

Crocodylians are gorge feeders and can experience a large alkaline tide following feeding (Coulson *et al.*, 1950; Weber and White, 1983). This relatively large alkaline tide could be a result of large amounts of gastric acid secretion in these animals. I recorded intragastric pH values for 6.5 days after feeding in two alligators and intragastric pH remained at around 1.5 to 2, which is the ideal pH for pepsin action in the stomach (Figure 1.9). Thus digestion is a long-lasting event in these animals. Flow to the lungs is not compromised in alligators during shunting periods as flow to the lungs occurs during early systole, and the ability to shunt for long periods of time after feeding may be a strategy to aid in the secretion of the large amounts of HCl needed in these animals and/or in ameliorating the large alkalosis that can be experienced in these animals. Jones (1996) proposed that shunting RV blood to the body may be used as a strategy to synthesize the large amounts of ammonium bicarbonate (NH_4HCO_3) excreted in these animals. Hydrated alligators primarily excrete their nitrogen waste as NH_4HCO_3 (Coulson and Hernandez, 1959; Coulson and Hernandez, 1964). CO_2 rich blood may be used to synthesize NH_4HCO_3 . In that case, RV blood would originate via the anastomosis because the kidneys are not directly perfused by the LAo. The anatomical

position of the anastomosis tends to support this suggestion (Figure 1.5). Also, our microsphere study showed that the kidneys primarily contained microspheres that were injected into the LAo during shunting periods (Figure 1.7). A third hypothesis proposed was that shunting blood high in CO₂ may be used to increase O₂ delivery to the tissues (White, 1978; Grigg and Johansen, 1987; Jones, 1996). The gut is a highly aerobic tissue and accounts for 20-35% of whole body oxygen consumption ($\dot{V}O_2$) during rest. Oxygen consumed by the gut increases following feeding (Takala, 1997). In humans, splanchnic $\dot{V}O_2$ increased 62 %, from 62 ml/min to 93 ml/min after ingesting fish protein (Brundin and Wahren, 1994). Also, haemoglobin oxygen affinity is reduced by CO₂ in crocodilians (Bauer and Jelkman, 1977; Bauer *et al.*, 1981; Grigg *et al.*, 1993; Jensen *et al.*, 1998). Shunting blood high in CO₂ to the active tissues (i.e. the digestive system) would aid in O₂ delivery during digestion when ATP turnover rates are high.

Recent studies have also investigated the role of shunting hypoxic blood to the body and inducing a hypometabolic state (Hicks and Wang, 1999; Platzack and Hicks, 2001). Alligators are opportunistic feeders and may go for long periods of time without food. Shunting RV blood to the gut may aid in conserving energy stores during times of fasting. Elevated blood and tissue CO₂ levels produce changes in pH and bicarbonate levels. Shunting blood high in CO₂ may also be a strategy to induce a hypometabolic state. For example, Malan *et al.* (1973) suggested that the relative acidosis experienced in marmots might inhibit metabolic or ionic transport processes to promote the induction of hibernation.

The goal of my thesis research was to determine the functional significance of the ability to shunt RV blood to the digestive system via the LAo in the American alligator (*Alligator mississippiensis*). The central hypothesis was that shunting RV blood to the digestive system aids in the synthesis of gastric acid (HCl) in the stomach and thus digestion and absorption of nutrients and/or by reducing the effects of the alkaline tide following feeding. Digestion affects acid-base status because of the net transfer of HCl from the blood to the stomach lumen. This leads to an increase in bicarbonate levels in the blood leaving the gut, i.e. the alkaline tide. Hydrochloric acid is secreted into the stomach by parietal cells in the gastric lining. CO₂ diffuses into the cell from the blood, forming H⁺ and HCO₃⁻, a reaction catalyzed by carbonic anhydrase. The HCO₃⁻ is transported across the basolateral membrane of the cell into the blood in exchange for an incoming Cl⁻ ion by means of an anion antiporter. Thus, shunting blood relatively high in CO₂ to the stomach may aid in the synthesis of HCl or in buffering the resulting increase in HCO₃⁻.

My prediction was that digestion and absorption would be impaired and prolonged in alligators that are unable to shunt RV blood to the digestive system via the LAo after feeding. Alligators without the ability to shunt RV blood to the digestive system via the LAo should have slower digestion rates and inadequate absorption of nutrients. I also predicted that alligators without the ability to shunt blood via the LAo would experience larger acid-base disturbances (more alkaline blood pH) following feeding.

To test my predictions and hypothesis, I used two groups of alligators. One group of alligators had their LAo surgically tied off and cut. These alligators were unable to shunt RV blood to the digestive system via the LAo. The second group had sham surgeries performed on them. These animals retained the ability to shunt RV blood to the digestive system via the LAo. I measured mass changes, metabolic rates, acid-base changes and blood variables during fasting and feeding. I also measured digestion rates, food intake and water intake during feeding. All variables measured are affected by digestion rate, digestion efficiency and digestive state. During fasting, shunting RV blood to the gut may aid in energy conservation by inducing a hypometabolic state of the tissues. Thus, alligators with their LAo intact should lose mass at slower rates during long-term fasting. I measured mass changes, metabolic rates, blood variables and acid-base changes to determine the effect of the ability to shunt RV blood to the digestive system during long-term fasting.

Figure 1.1. Two-dimensional drawing of a non-crocodilian reptile heart. CA = cavum arteriosum; CP = cavum pulmonale; CV = cavum venosum; SV = sinus venosus; PA = pulmonary artery; RAo = right aorta; LAo = left aorta; PV = pulmonary veins; RA = right atrium; LA = left atrium. The striped area represents the horizontal septum. Red arrows represent left atrial blood returning from the lungs; blue arrow represents right atrial blood returning from the body. Adapted from Heisler *et al.*, 1983.

Figure 1.2. Intracardiac shunting due to the washout mechanism in a varanid. CA = cavum arteriosum; CP = cavum pulmonale; CV = cavum venosum; SV = sinus venosus; PA = pulmonary artery; RAo = right aorta; LAo = left aorta; PV = pulmonary veins; RA = right atrium; LA = left atrium. The striped area represents the horizontal septum. Red represents oxygenated blood returning from the lungs; blue represents deoxygenated blood returning from the body. Adapted from Heisler *et al.*, 1983.

Figure 1.5. Drawing of the crocodilian cardiac anatomy and photograph of the anastomosis of an alligator (modified from Doug Syme). The point at which the LAo was ligated is shown by the thick blue line.

Figure 1.6. Arteries of the posterior region of an alligator with special attention to the left aorta (LAo) and its branches (modified from Reese, 1915).
 RAo – right aorta. Red – signifies left ventricular blood; Blue – signifies right ventricular blood.

Figure 1.7. Regional blood flow distribution during non-shunting and shunting periods for alligators. RAo - right aorta; LAo - left aorta. Significant differences between injection sites are indicated with an asterisk. Non-shunting periods: RAo injection - N = 4; LAo injection - N = 3. Shunting periods: RAo injection - N = 2; LAo injection - N = 2.

Figure 1.8. Arteries of the posterior region of *Crocodilus niloticus* (modified from Bolk *et al.*, 1967). RAo – right aorta. LAo – left aorta.

Figure 1.9. Intra-gastric pH changes (blue trace) and temperatures (red trace) in two alligators (1 and 2) after feeding. The probe was given to each alligator at time "0". A: The first 24 hours after feeding indicating time of feeding. B: The whole recording period in each alligator with a total time of 6.5 days.

CHAPTER 2

Growth, digestion and metabolic rates during feeding in alligators

Introduction

Crocodylians are ferocious feeders. They swallow every part of their prey and thrive on a high protein diet (Coulson *et al.*, 1987). Protein digestion is started in the stomach by the proteolytic enzyme pepsin which hydrolyses protein into large polypeptides and these are further broken down into amino acids within the intestine. A pH of 1.5 to 3.5 within the stomach is required in order for pepsin to operate optimally. Consequently, gastric acid (HCl) secretion is very important for the initial stages of protein digestion in the stomach, and large amounts of gastric acid may be necessary to digest protein in crocodylians due to their diet.

Gastric acid secretion is controlled by both neural (vagus nerve) and hormonal (gastrin, histamine) mechanisms. HCl production by the parietal cells is dependant upon the enzyme carbonic anhydrase (CA) which catalyzes the formation of carbonic acid (H_2CO_3) in the cell ($\text{H}_2\text{O} + \text{CO}_2 \leftrightarrow \text{H}_2\text{CO}_3$). Carbonic acid dissociates into protons (H^+) and bicarbonate (HCO_3^-) and H^+ is pumped into the stomach lumen in exchange for potassium (K^+). The H^+ concentration in parietal cells is about 10^6 times higher than in the blood and during acid secretion H^+ is pumped against this large concentration gradient via the H^+/K^+ ATPase (proton pump). Chloride (Cl^-) is exchanged for HCO_3^- at the basolateral side and is transported into the stomach lumen with K^+ by conductance channels. A study by Coulson (1985) showed that when CA is inhibited, HCl production

is reduced and protein digestion delayed in alligators. In that study, peak values in amino acid appearance (used as an indicator of digestion rates) were detected thirty hours after feeding and the amount of HCl was 12 mmol/kg in controls (no CA inhibition) and only 4 mmol/kg in alligators that were injected with the CA inhibitor dichlorphenamide. In controls, digestion (as indicated by an increase in plasma amino acids) began immediately but was delayed for 10 hours in alligators in which CA was inhibited.

Digestive efficiency and digestion rates are affected by the ability to adequately break down food in the stomach and these in turn affect growth rates. Compromising the initial stage of protein digestion in the stomach as a result of inadequate HCl secretion affects absorption and assimilation of protein and thus protein synthesis. This may in turn affect somatic growth rates in young alligators.

Digestion in reptiles can be a prolonged process taking many days or even weeks (Gatten, 1980; Davenport *et al.*, 1990; Secor, 2003) and HCl secretion by the parietal cells is an energetically demanding process (Helander and Keeling, 1993). For example, the large increase in oxygen consumption rates ($\dot{V}O_2$) to feeding in pythons was largely due to increased gastric function. Secor (2003) found that the production of HCl, enzymes associated with digestion and other gastric functions represented about 55% of the python's increase in metabolic rate (measured as an increase in $\dot{V}O_2$) in response to ingesting 25% of total body mass in intact rats. Also, intragastric pH remained below 2.0 in these pythons for one week after feeding and $\dot{V}O_2$ of the snakes was elevated above resting levels for 10 days. We similarly found that for alligators intragastric pH was

around 1.5 - 2.0 for 6.5 days after ingesting 4% of total body mass of whole rats (Figure 1.9).

Following feeding $\dot{V}O_2$ in the splanchnic regions increases. This results in an increase of blood flow (postprandial hyperemia) to the active tissues via local metabolic products of cell metabolism such as CO_2 , local reflexes related to the presence of food and locally produced vasoactive substances such as the hormones gastrin and secretin. Extrinsic changes include input from sympathetic innervation, circulating vasoactive substances such as catecholamines and systemic hemodynamic changes such as an increase in cardiac output (Granger *et al.*, 1980).

In dogfish and frog, acid secretory rate by the gastric mucosa in vitro was limited by CO_2 availability (Kidder and Montgomery, 1974; Kidder, 1976). The LAo of alligators supplies blood to the gastrointestinal system (GI tract) and digestion takes a long time in these animals. Also, alligators may shunt as much as 85% of the time when calm which could be a manner exhibited during digestion (Jones *et al.*, in preparation). Thus, shunting RV blood to the digestive system following feeding may aid in the secretion of HCl by supplying the stomach with relatively acidic blood (Jones and Shelton, 1993). To test the hypothesis that the ability to shunt RV blood via the LAo to the stomach aids in HCl secretion and thus protein digestion, this study investigated the effect of ligating the LAo of young alligators on growth rates (measured as mass and length increases), digestion rates, food conversion rates (mass gained/mass consumed) and metabolic rates during feeding compared with sham operated controls. One group of alligators had their LAo surgically tied off and cut and were unable to shunt RV blood

via the LAo. The other group had sham surgeries performed on them and retained the ability to shunt RV blood via the LAo. We predicted that alligators with their LAo ligated would grow at slower rates, have lower food conversion rates, have slower digestion rates, and have different metabolic states which would be reflected by higher RER values due to slower digestion rates.

Material and Methods

Animal care and maintenance

Alligators (*Alligator mississippiensis*) were purchased from a commercial alligator farm in Florida and shipped by air to Vancouver, British Columbia. The animals were housed together in a large room with a shallow pool that was flushed with continuously flowing water at the University of British Columbia (UBC) animal care facility. Alligators were able to freely move around the room at all times. Water temperature was kept at $29.9 \pm 0.8^{\circ}\text{C}$ (mean $\pm$ SD) and average room temperature was $26.0 \pm 2.2^{\circ}\text{C}$. A 12 hour light and 12 hour dark photoperiod was maintained throughout the study using full spectrum lights. Alligators were fed pieces of chicken, fish or whole mice and rats supplemented with multivitamins.

Surgical procedures

All animals were fasted at least 48 hours before surgery. Alligators were initially anesthetized with a gas mixture of 4% isoflurane in oxygen using a facemask. A surgical level of anesthetic was maintained by inhalation of 0.8% - 2% isoflurane via an

endotracheal tube. In young animals the sternum is not ossified, so a small incision (about 3 cm) was made at the ventral midline to expose the heart and to locate the major vessels exiting the heart. The left aorta (LAo) was tied off and cut just outside the heart in one group of alligators (Figure 1.5). Sham surgeries were performed on another group of alligators in which the blood vessels were exposed and identified but the LAo was left intact. At the end of surgery, animals were ventilated with 100% oxygen until fully awake and alert. The animals were put into a dry room for 48 hours. They were then put back into their room with the water tank. This aided in the healing process. Baytril (antibiotic) was given intramuscularly every three days for one week to prevent any post-surgical infection of the incision. All alligators were left alone for at least 4 weeks before further studies were done.

All surgical procedures and experiments were approved by the University of British Columbia Committee on Animal Care.

A note on working with alligators

Working long-term with alligators posed several challenges and these affected the results of my study and caused some inconsistencies in my measurements. Alligators are very social animals and get stressed and refuse to eat when isolated. Even if force-fed they often regurgitate their food. Handling also upsets alligators, even if they have been handled on a daily basis from early in life. This will affect measurements such as blood variables. For example, taking several consecutive blood samples becomes increasingly difficult due to the increased stress level of the alligators. Also, stressed alligators are

more prone to getting sick. I tried to minimize stress in my alligators because many of my measurements were done long-term (months). However, this prevented me from measuring some blood variables as frequently as I would have liked and from attaining certain measurements such as exact food intake.

Feeding

Eight month old alligators were fed *ad lib* daily for four months after surgery. Initially they were fed a maximum of 25% of total body mass of food per group per week, but later food was restricted to 5% of total body mass of food per group per week.

To allow determination of the exact amount of food eaten by each group, the two groups of alligators were separated during feeding. All alligators were weighed before and after feeding. After weighing all the alligators, each group was fed an equal amount of food as a percentage of total body mass per group for an equal amount of time. Leftover food was removed and weighed, so that food intake per group could be recorded.

Water intake during feeding can be large in alligators and thus can affect mass measurement following feeding. This is an important factor to consider when weighing animals following feeding, especially if the exact amount of food eaten is not known. Measurement of water intake is important because the gastric environment is affected by diluting it. Water intake for the two groups of alligators was determined by weighing the alligators after feeding. Knowing the food intake for each group, water intake could be calculated by subtracting the amount of food eaten per group (g) from the total amount of

mass gained per group. For studies of digestion rates and respiratory variables we were not able to separate our alligators during feeding, so the exact amount of food eaten or water consumed could not be determined.

Growth rates

Eight month old alligators were used for studies of growth rates. Mass (g) and total length (measured as tip of snout to tip of tail (cm)) were measured each week for all alligators.

Blood sampling

Each alligator was removed from the group and the mouth was taped shut. The procedure of getting the alligator, taping the mouth and taking the blood was timed using a stop watch because measurements such as blood PCO_2 , PO_2 and pH were affected by elapsed sampling time. Blood was taken from the cervical sinus in the neck region using a sterile 1.5 inch, 20 gauge needle into a 5 ml syringe. Depending on the experiment, different amounts of blood were taken. The maximum single amount taken was 5 ml per alligator. After taking the blood from the alligator, 1 ml of whole venous blood was slowly injected into the pH/blood gas analyzer (Chapter 4). Hematocrit of the sampled blood was measured using hematocrit tubes. Depending on the experiment, the rest of the blood was put into heparinized vacutainer plasma tubes (Becton Dickinson, USA) or serum separation tubes (SST; Becton Dickinson, USA) and put on ice. All the tubes were centrifuged within 30 minutes of blood sampling for 10 minutes at 3000 rpm. The

plasma or serum was aliquoted with a 1 ml syringe and stored in cryo-safe tubes (Starstedt Inc., Montreal, QC) at -8° C until further analysis.

Digestion rates

Digestion rates were measured using two methods: (1) by measuring the rate of amino acid appearance in plasma following feeding and (2) by using Barium Impregnated Polyethylene Spheres (BIPS) which allow calculation of retention time of food in the gastrointestinal tract.

The increase in plasma amino acids following feeding can be used as an indicator of digestion rate because the digestion of proteins and subsequent absorption of amino acids from the small intestine is directly proportional to digestion rate (Coulson *et al.*, 1950; Coulson and Hernandez, 1967; Coulson and Coulson, 1986; Coulson *et al.*, 1987). Plasma amino acid concentration was analyzed just before feeding, 25 hours, 72 hours and 121 hours after feeding. These intervals were based on previous studies of amino acid appearance after feeding (Coulson *et al.*, 1950; Coulson and Hernandez, 1967; Coulson and Coulson, 1986; Coulson *et al.*, 1987).

This study was done on an older (3 years of age) group of alligators. Due to limited availability of space, the two groups of alligators could not be separated during feeding and so exact food intake could not be determined for each group for this study. Alligators were fed weekly and thus the first blood sample was taken 7 days after the last feeding (pre-feeding sample). All alligators were weighed following the initial blood sample prior to feeding and then fed *ad lib*. Alligators were weighed again after the first

post-feeding sample i.e. 25 hours later. This allowed us to approximate food intake, including water consumed. The animals could not be weighed immediately after feeding because the animals get very upset during blood sampling and often regurgitate any recently eaten food. After blood sampling and weighing, alligators were returned to the group.

Plasma amino acid concentrations were measured in 1 ml of whole venous blood. Plasma amino acids were determined using Danielson's method (1933; in Hawks Physiological Chemistry, 1965). A protein-free filtrate was prepared using the method of Folin and Wu (1919; in Hawks Physiological Chemistry, 1965). Amino acid concentration was determined spectrophotometrically by comparing the sample absorbance to a standard with a known amino acid concentration. Plasma amino acids were calculated as mg% (mg amino acid nitrogen per 100 ml blood).

Barium impregnated polyethylene spheres (BIPS) are inert, white, low density radiopaque spheres. Large BIPS (5.0 mm diameter) are used to determine obstructions within the gastrointestinal (GI) tract (Allan *et al.*, 1996; Chandler *et al.*, 1997; Nelson *et al.*, 1996). Small BIPS (1.5 mm diameter) have a density similar to food and show up clearly on abdominal radiographs. Hence, detection of BIPS by taking x-rays at different intervals allows calculation of transit time of food through the GI tract.

This study was done on the 8 month old alligators used for growth rate measurements. Due to constraints at the x-ray facility (UBC Radiology; University of British Columbia, Vancouver, Canada) we were only able to feed BIPS to one alligator

from each group per week. All the alligators in each group were used for this study. Unfortunately, we were unable to determine the exact food intake per alligator as individual alligators from either group were not isolated. Each alligator received 30 small BIPS during feeding. The BIPS were embedded into the food, which was fed to each alligator at the beginning of the feeding session with large tweezers. Alligators took BIPS containing food freely from the tweezers. After alligators had been fed food containing the BIPS they were allowed to feed *ad lib* the allotted amount of food, i.e. 5 % of total body mass of food per group. Alligators in each group were given an equal amount of time to eat their food and were weighed again after feeding.

The alligators were taken to UBC Radiology at different intervals to determine transit time of food through the GI tract. Again, the intervals chosen were based on previous studies of amino acid appearance following feeding (Coulson *et al.*, 1950; Coulson and Hernandez, 1967; Coulson and Coulson, 1986; Coulson *et al.*, 1987) and on the availability of the x-ray equipment at UBC Radiology and were 18-21 hours, 94-95 hours and 142-144 hours after feeding. During x-ray imaging all alligators had their mouths taped and heads covered with a cloth. No additional restraint was necessary. After the x-rays were taken the alligators were returned to the holding facility.

Oxygen consumption and carbon dioxide release

Rates of oxygen consumption ($\dot{V}O_2$) and carbon dioxide release ($\dot{V}CO_2$) were measured during feeding and fasting (see chapter 3) using open-circuit respirometry. The metabolic chamber was a clear bottomless Plexiglas box that was submerged under

water in the large water tank in the alligator holding room so animals did not have to be removed from their normal surroundings to measure respiratory variables. Holes in the chamber minimized water pressure fluctuations due to animal movements. A funnel attached to the top of the chamber received a continuous flow of air during the experiment and served as the respiratory chamber for the alligator. To ensure familiarity with the setup and procedure, alligators were put into the chamber 24 hours before recording for a duration equal to that of the experiment. Disturbance was kept to a minimum by not entering the room during the recording period.

A Li-Cor carbon dioxide analyzer (Li-Cor, Inc., USA) and Ametek oxygen analyzer (Ametek, USA) were used in series to record CO₂ and O₂, respectively. A subsample of the excurrent air was pumped through a column of magnesium perchloride (drying agent) upstream of the Li-Cor CO₂ analyzer and through a column of drierite (drying agent) and soda lime (to remove CO₂) upstream of the oxygen analyzer. The Ametek O₂ analyzer and Li-Cor CO₂ analyzer were calibrated using a pre-calibrated gas mixture (Praxair, Vancouver, BC) before each trial and immediately following an experiment to check for changes in baseline due to electronic drifts. Oxygen and CO₂ concentrations were continuously recorded on a computer using a data acquisition program (Labtech, USA). In the output file, downward deflections from baseline represented changes in O₂ concentration and upward deflections from baseline represented changes in CO₂ concentration. The respirometry system was checked for stability and leaks by injecting known quantities of nitrogen into the funnel after each experiment.

$\dot{V}O_2$ and $\dot{V}CO_2$ were measured on the older (3 years) and larger group of alligators. Measurements were taken 6 days after feeding. The two groups of alligators could not be separated during feeding, so measurements of food intake included water intake. The average mass was 8.78 ± 0.95 kg (Mean $\pm$ SEM) and 4.06 ± 0.40 kg for alligators with their LAo intact (N = 3; n = 6 where N is the number of animals used and n is the number of experimental trials per group of animals) and alligators with their LAo cut (N = 3; n = 7), respectively. The large difference in body mass was due to the addition of new alligators to the LAo intact group to replace alligators that died due to fatal bites or stress related infections. These alligators had sham surgeries performed on them and were allowed to recover for at least 4 weeks following surgery.

Data analysis and statistics

Statistical analyses were performed using JMP (SAS Institute, Inc.) and SigmaStat software (Jandel Corporation, USA). Unpaired t-tests were used to test for significant differences between groups. An Analysis of Covariance (ANCOVA) was used to test for significant effects of body mass, treatment (LAo cut) and food intake on measured variables. A significant difference was accepted at the level of $P < 0.05$. All values are presented as Mean $\pm$ Standard error of the Mean (SEM).

Growth rates

Growth rates were averaged for each group of alligators. Due to differences in size, growth rates and food intake were normalized for body mass. Growth rates were

recorded as % change in mass per group per week $((M2 - M1) * 100) / M1$; where M1 was the total mass per group the previous week and M2 was the total mass per group by the next week). Food intake was measured as food eaten as a % of total body mass per group per week $((g \text{ of food eaten} * 100) / \text{total mass of group})$. Conversion rates or digestive efficiencies were calculated from the growth rates. Conversion rates refer to the amount of food converted into new mass. Knowing the amount of food eaten in each group (g) and the increase in mass in each group (g), conversion rates were calculated as a % of food eaten. Water intake was calculated by subtracting the total amount of food eaten per group (g) from the total amount of mass gained by each group (g) after feeding.

Digestion rates

Levels of amino acids following feeding were averaged for each time interval at which blood was sampled in both groups of alligators.

Transit time of food through the GI tract was calculated by counting the small BIPS within the GI tract at the different intervals when x-rays were taken. The values were averaged for each group of alligators. Food intake (mass gained after feeding plus water intake) was calculated as a % mass gained per alligator and averaged per group.

Oxygen consumption and carbon dioxide release

Oxygen consumption and carbon dioxide release were recorded for 5.40 ± 0.09 hours (Mean $\pm$ SEM). $\dot{V}O_2$ was determined by measuring the area under the downward deflections from the baseline reading (ambient O_2 concentration) and $\dot{V}CO_2$ was

determined by measuring the area under the upward deflections from the baseline reading (ambient CO₂ concentration) using a data analysis program (Acqknowledge, BIOPAC Systems, Inc.). Data were averaged over the entire period, except for the first hour. This allowed for an additional familiarization period of the alligator with the chamber while the recording apparatus was calibrated and set-up was completed. Due to the significant difference in mass during feeding between the two groups of alligators, an ANCOVA was performed on $\dot{V}O_2$, $\dot{V}CO_2$ and respiratory exchange ratios (RER) with mass as a covariate to determine significant differences between groups. Significance was set at $P < 0.05$. All values were corrected to STPD.

Results

Growth and conversion rates

Figure 2.1 shows the relationship between length (cm) and mass (g) in the two groups of alligators (LAo intact: N = 5; n = 360; LAo cut: N = 6; n = 432). Both groups of alligators showed similar length-mass relationships.

Food intake and corresponding weekly growth rates are shown in Figure 2.2. When alligators were fed *ad lib*, food intake ranged from 10 % to 23 % of total body mass (average 15.2 ± 1.0 %) per week for alligators with their LAo intact and from 10 % to 25 % (average 17.5 ± 1.1 %) per week for alligators with their LAo cut (Figure 2.2). Average conversion rates were 35.9 ± 4.4 % for alligators with their LAo intact and 38.9 ± 4.7 % for alligators with their LAo cut. When alligators were fed 5 % of total body mass per group per week, average food intake was 4.87 ± 0.05 % and

4.77 ± 0.06 % per group per week and corresponding group average growth rates were 1.70 ± 0.14 % mass change per week and 1.72 ± 0.13 % mass change per week for alligators with their LAo cut and alligators with their LAo intact, respectively. Conversion rates were 36.5 ± 2.3 % and 34.8 ± 2.9 % for alligators with their LAo cut and LAo intact, respectively. There was no significant difference in food intake or conversion rates between groups.

The average weekly mass measurements for the two groups of alligators before surgery, after surgery when alligators were fed a maximum amount of food of 25 % per group per week and later when alligators were fed 5% of food per group per week are shown in Figure 2.3. The average mass between the two groups of alligators was not significantly different when measurements were taken before surgery or after surgery when alligators were fed *ad lib* (Figure 2.3 B). Alligators with their LAo cut were significantly larger during the period when fed 5 % of food per group per week. Average growth rates were not significantly different during that period.

Water intake was variable following feeding, but was not different between the two groups of alligators (Figure 2.4). There was no correlation between food intake and water intake. Food intake was not significantly different between the two groups of alligators (Figure 2.2).

Digestion rates

The number of BIPS within the GI tract was not different between the two groups of alligators (Figure 2.5). The average gain in mass following a meal containing BIPS

was not different between the two groups of alligators ($7.0 \pm 1.5 \%$ and $7.8 \pm 0.5 \%$ for alligators with their LAo intact and with their LAo cut, respectively). There was no significant difference between the average mass for alligators with their LAo intact ($1.75 \pm 0.38 \text{ kg}$) and those with their LAo cut ($2.76 \pm 0.32 \text{ kg}$).

Figure 2.6 shows average plasma amino acids levels following feeding in the two groups of alligators. Average mass of these alligators during this experiment was $6.8 \pm 0.9 \text{ kg}$ and $4.2 \pm 0.5 \text{ kg}$ for alligators with their LAo intact ($N = 7$; $n = 20$) and LAo cut ($N = 6$; $n = 28$), respectively. There was a significant difference in mass between the groups ($P = 0.026$), but mass did not have a significant effect on plasma amino acid measurements as determined by ANCOVA ($F = 0.66$, $P = 0.426$). At 25 hours after feeding, amino acid levels were significantly higher in alligators with their LAo intact. However, food intake (including water intake) during the study was significantly higher in alligators with their LAo intact ($4.12 \pm 0.72 \%$ versus $2.79 \pm 0.42 \%$ of body mass). Statistical analysis confirmed that the significant difference at 25 hours was due to the difference in food intake ($F = 3.30$; $P = 0.005$).

Oxygen consumption and carbon dioxide release

Average gas exchange values are given in Table 2.1. Average $\dot{V}O_2$ values were not significantly different ($0.038 \pm 0.003 \text{ ml/g/hr}$ for alligators with their LAo intact and $0.032 \pm 0.003 \text{ ml/g/hr}$ for alligators with their LAo cut). However, there was a significant effect of mass on $\dot{V}O_2$ values ($F = 6.1$, $P = 0.035$). Average $\dot{V}CO_2$ values were significantly higher in alligators with their LAo cut ($0.021 \pm 0.002 \text{ ml/g/hr}$)

compared with alligators with their LAo intact (0.014 ± 0.001 ml/g/hr). This significant difference was due to the effect of mass and food intake on $\dot{V}CO_2$ (mass: $F = 44.026$, $P < 0.0001$; food intake: $F = 14.81$, $P = 0.027$). Average food intake (including water intake) was not significantly different in alligators with their LAo cut (2.5 ± 0.91 %) compared with alligators with their LAo intact (4.6 ± 1.4 %). Average RER values were significantly higher in alligators with their LAo cut (Table 2.1; Figure 2.7). Average RER values were 0.64 ± 0.02 and 0.39 ± 0.04 for alligators with their LAo cut and alligators with their LAo intact, respectively. The significant difference in RER values between the two groups of alligators was due to the effect of mass, i.e. alligators with their LAo intact were significantly larger ($F = 7.9$, $P = 0.017$). Statistical analysis showed that the amount of food eaten had no significant effect on RER values ($F = 0.2$, $P = 0.66$).

Discussion

Crocodylians are very efficient at digesting and utilizing protein and juvenile crocodiles can have protein digestibility of as high as 86.7% (Staton *et al.*, 1990). Compromising protein digestion and absorption in young crocodylians greatly affects growth. Davenport *et al.* (1990) found greatly reduced growth in one *Crocodylus porosus* whose digestion and absorption of protein was inadequate. This crocodile showed appetite but had a slower rate of digestion with assimilation of protein nitrogen being only one-third that of the others. Why protein digestion and absorption were low in this crocodile was not determined. We predicted that growth rates should be slower in

alligators without the ability to shunt blood via the LAo to the digestive system. We found that for a given amount of food eaten, growth rates varied within each group but no significant difference was found between the two groups of alligators (Figures 2.1, 2.2 and 2.3). Similar growth rates imply that digestion rates, absorption of nutrients and digestion efficiencies/conversion rates were also similar. Digestion rates were not significantly different between the two groups of alligators (Figures 2.5 and 2.6). The appearance of a significant difference in average amino acids in the blood at 25 hours post-feeding was, in fact, due to variability in food intake between the two groups of alligators. We also did not see a significant difference in water intake between the two groups of alligators (Figure 2.4), nor was there a correlation between water intake and food intake. Consequently, any effect of drinking water on digestion should have been similar in the two groups of alligators.

Alligators with their LAo cut were significantly heavier when fed 5 % of total body mass of food per group per week (Figure 2.3). The difference in mass was due to two alligators that were much smaller in the group with their LAo intact. However, the average amount of food eaten and average growth rates were not significantly different between the two groups of alligators. We calculated the conversion rates based on growth rates in the two groups of alligators and again found that these were not different. Conversion rates have been measured in crocodilians and a range of values have been found. These values depend on body temperature of the animal, age, hydration and type and amount of food eaten (Coulson *et al.*, 1973; Joanen and McNease, 1987; Davenport *et al.*, 1990; Staton *et al.*, 1990; Webb *et al.*, 1990; Webb, 1991). For example, Webb *et*

al. (1990) found conversion rates of 28 % in the first month of life in young *C. porosus* fed daily. The conversion rates increased to 40 % when feeding occurred every two days. So, even the feeding regime can have an impact on conversion rates. When these animals were fed daily, their stomachs were always full and so the physiological mechanisms associated with digestion in the stomach (physical and chemical breakdown of food) may be inhibited. Studies on American alligators have shown conversion rates of 25-40 % (Coulson *et al.*, 1973; Joanen and McNease, 1987). Our conversion rates ranged from about 35 % to about 39 % for both feeding daily *ad lib* and feeding 5% of total body mass per group per week and so the feeding regime did not significantly affect conversion rates. This could be a result of having full stomachs when fed daily and thus digestion and absorption of food is not increased with an increase in food intake. Thus, our results indicate that the ability to shunt RV blood to the digestive system via the LAo does not aid in digestion and subsequent absorption of food in alligators.

Indirect calorimetry can be used for estimating an animal's metabolic energy expenditure based on rates of oxygen consumption ($\dot{V}O_2$) or carbon dioxide production ($\dot{V}CO_2$) measured across the lungs and the ratio of $\dot{V}CO_2/\dot{V}O_2$ is defined as the respiratory quotient (RQ). Due to the possibility of non-pulmonary loss of CO_2 , measured $\dot{V}CO_2$ values may not reflect tissue metabolism and thus the term respiratory exchange ratio (RER) will be used. Depending on the fuel metabolized (fats, proteins or carbohydrates) mammalian RER values typically range from 0.7 during fasting to 1.0 for a high carbohydrate diet. The data on metabolic rates in crocodilians varies widely, with

$\dot{V}O_2$ values from 0.018 ml/g/hr to 0.09 ml/g/hr (Anderson, 1961; Smith, 1975; Grigg, 1978, Gatten, 1980; Lewis and Gatten, 1985; Garnett, 1986; Busk *et al.*, 2000; Farmer and Carrier, 2000). Farmer and Carrier (2000) reported $\dot{V}O_2$ values of 0.018 ml/g/hr and $\dot{V}CO_2$ values of 0.0138 ml/g/hr in alligators at 30° C (average mass 1.34 kg) 4 days after feeding. Our $\dot{V}O_2$ values were higher for both groups of alligators but $\dot{V}CO_2$ values were similar (0.014 ml/g/hr). Another study (Busk *et al.*, 2000) found similar $\dot{V}O_2$ values to ours (0.038 ml/g/hr) but higher $\dot{V}CO_2$ values (0.026 ml/g/hr) in alligators that were fasted for 3-4 weeks. Our alligators with their LAo cut had average $\dot{V}CO_2$ values of 0.021 ml/g/hr which are similar to those found in the study by Busk and colleagues (2000). The variability of the data may be due to numerous factors such as the type of food eaten prior to making measurements, body temperature of the animal, stress level, activity, size, and time of day. The digestive state of the animal may also have a significant effect on metabolic rate. The impact of digesting food on metabolic rates is significant and can vary greatly within the same group of animals (Gatten, 1980; Coulson *et al.*, 1990). In crocodilians, digestion can take a long time. For example, Davenport *et al.* (1990) showed that total gut clearance took 4 to 5 days in very young crocodiles with a mean mass of around 154 g. Gatten (1980) used x-rays to confirm that full digestion of food took 2-3 weeks in one caiman. Gatten's caimans had a mean mass of 1684 g, so they were also young animals. We have found food within the gut of alligators one month after the onset of fasting (unpublished data). Thus, it is important to know the digestive state and amount and type of food eaten by an animal when recording metabolic rates, especially when comparing different groups of animals.

Average RER and $\dot{V}CO_2$ values were significantly higher in alligators without the ability to shunt via the LAo. Average RER values for both groups fell within the range of RER values reported by Grigg (1978) (0.32 to 0.74). Grigg measured metabolic rates in *Crocodylus porosus* 3 days after capture and the digestive state was unknown. In our study, for a given amount of food eaten, RER values were always higher in alligators with the LAo cut. Even when an alligator did not eat for 13 days, RER values were higher in alligators with the LAo cut. However, significant differences in $\dot{V}CO_2$ and RER values between the two groups of alligators were due to the effect of mass and food intake on $\dot{V}CO_2$ and not to the ability to shunt via the LAo.

One surprising result was the unusually low RER values in our alligators. "Typical" RER values are not necessarily seen in reptiles (see Hall, 1924; Potter and Glass, 1931; Cook, 1949; Smith, 1975; Grigg, 1978; Stinner *et al.*, 1996). Grigg (1978) suggested that crocodilians might use respiratory CO_2 to buffer urinary ammonia. Alligators primarily excrete ammonium bicarbonate (NH_4HCO_3) when hydrated and average urine pH is around 7.8 (Coulson and Hernandez, 1959; Coulson and Hernandez, 1964). Also, some bicarbonate may be excreted through the feces (Atkinson and Bourke, 1984). If respiratory CO_2 is used to synthesize NH_4HCO_3 in alligators, measured RER values could be lower than expected and not reflect expected tissue metabolism. Can NH_4HCO_3 synthesis cause low RER values? Grigg (1979) did some calculations that can be applied to our study. In our alligators, average $\dot{V}O_2$ values were 16.9 L/day with their LAo intact (average mass 8.78 kg) and 3.1 L/day for alligators with their LAo cut (average mass 4.06 kg). Assuming a "normal" RER of about 0.8 (indicative of protein

catabolism), $\dot{V}CO_2$ should have been about 13.5 L/day for alligators with their LAo intact and 2.5 L/day for alligators with their LAo cut. We know that 960 ml O_2 are required to catabolize 1 g protein and that this produces about 0.16 g of nitrogenous wastes. So, in alligators with their LAo intact 2.81 g of nitrogen were produced per day (based on their daily $\dot{V}O_2$) and in alligators with their LAo cut 0.52 g of nitrogen were produced per day. This would require 0.2 mol of CO_2 as bicarbonate for alligators with their LAo intact (14 g nitrogen/mol) and 0.04 mol CO_2 for alligators with their LAo cut. This would represent 4880 ml CO_2 excreted in alligators with their LAo intact (24.4 l/mol) and 976 ml CO_2 excreted in alligators with their LAo cut. So, the RER for each would be:

$$RER = (\dot{V}CO_2 - CO_2 \text{ as nitrogenous waste}) / \dot{V}O_2$$

Thus, $(13.5 - 4.88)/16.9 = 0.51$ for alligators with their LAo intact and $(2.5 - 0.98)/3.1 = 0.49$ for alligators with their LAo cut. The calculated RER values based on nitrogen excretion alone agree closely with measured RER's and thus nitrogen excretion in our alligators may have resulted in lower than expected RER values. Also, Coulson and Hernandez (1983) stated that at rest a 1 kg alligator may excrete as much as 22% of its CO_2 via the kidney and this percentage could increase when alligators are fed maximally. Following feeding, metabolic CO_2 may also be used in the synthesis of HCl ($CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$) and sodium bicarbonate in the pancreas ($NaHCO_3$) and this can also lower measured $\dot{V}CO_2$ values.

We measured respiratory variables 6 days after feeding. Amino acid appearance seemed to be completed at 72 hours after feeding (Figure 2.6) but more than 20 BIPS were still in the stomachs of our alligators after 6 days (Figure 2.5), suggesting a low level of digestion. Our alligators were fed weekly and pre-feeding amino acid values

were measured 7 days after the previous week's feeding. So, pre-feeding amino acid levels may not necessarily represent true fasting levels. For example, a study by Coulson and Coulson (1986) showed that plasma amino acid levels stayed elevated for over 100 hours at 31° C in alligators weighing from 780 g to 1080 g that were fed 5% of food per total body mass. So, the difference between the two methods used to measure digestion rates may be explained by differences in sampling technique.

We hypothesized that the ability to shunt RV blood to the digestive system following feeding may aid in HCl secretion and thus protein digestion which in turn would affect digestion rates, conversion rates and growth rates. However, growth rates, conversion rates and digestion rates were not affected by the ability to shunt RV blood to the digestive system via the LAo. Alligators with their LAo intact may shunt RV blood to the body via the LAo to buffer the metabolic alkalosis experienced following feeding. Vertebrates adjust their ventilation to changes in acid-base status in order to maintain homeostasis and this can be reflected in measured respiratory variables such as $\dot{V}CO_2$. After feeding, the increase in $\dot{V}CO_2$ is proportionally less than the increase in $\dot{V}O_2$ in alligators and amphibians to compensate for the metabolic alkalosis experienced after feeding (Chapter 4; Busk *et al.*, 2000a, b). Again, this would be reflected in lower than expected $\dot{V}CO_2$ values and thus lower than expected RER values. Shunting relatively acidic blood to the body may aid in ameliorating the alkaline tide and this will be investigated in Chapter 4.

Figure 2.1. Length-mass relationship of two groups of alligators. LAo intact: N=5; LAo cut: N=6.

Figure 2.2. Food intake per group of alligators per week and the corresponding % change in mass per group per week. LAo intact: N=5; LAo cut: N=6.

Figure 2.3A and B. A: Weekly average mass measurements (g) in two groups of alligators before surgery, after surgery being fed *ad lib* and after surgery being fed 5% of food per group per week. LAo intact: N=5; LAo cut: N=6. Values are shown as means $\pm$ SEM. For clarity, only positive SEM are shown for alligators with their LAo cut and for alligators with their LAo intact only negative SEM are shown. B: Average mass in two groups of alligators during each feeding period. Values are shown as means $\pm$ SEM. Significant differences between groups are indicated with an asterisk.

Figure 2.4. Relationship between g of food eaten and water intake (g per group) in two groups of alligators. LAo intact: N=5; LAo cut: N=6.

Figure 2.5. The number of BIPS within the GI tract of two groups of alligators following feeding. LAo intact: N=5; LAo cut: N=6. All values are Means $\pm$ SEM.

Figure 2.6. Change in plasma amino acids appearance (mg amino acid nitrogen per 100 ml blood) following feeding. LAo intact: N=7; LAo cut: N=6. Values are shown as means $\pm$ SEM. Significant differences between groups are indicated by an asterisk.

Figure 2.7. Respiratory variables during feeding in two groups of alligators. $\dot{V}O_2$, rate of oxygen consumption; $\dot{V}CO_2$, rate of carbon dioxide release; RER, respiratory exchange ratio; % food intake per total body mass per alligator versus RER values. LAo intact: N=3; LAo cut: N=3.

Table 2.1. Average $\dot{V}O_2$, $\dot{V}CO_2$ and RER values in two groups of alligators 6 days after feeding.

	$\dot{V}O_2$ (ml/g/hr)	$\dot{V}CO_2$ (ml/g/hr)	RER	Food intake (%)
LAo intact	0.038 ± 0.003	0.015 ± 0.001	0.395 ± 0.037	4.6 ± 1.4
LAo cut	0.032 ± 0.003	0.021 ± 0.002	0.638 ± 0.019	2.5 ± 0.91

Legend:

$\dot{V}O_2$: oxygen consumption rates; $\dot{V}CO_2$: carbon dioxide release; RER: respiratory exchange ratio; Food intake: % of total body mass.

LAo intact during feeding: N=3; LAo cut during feeding: N=3.

CHAPTER 3

Rate of mass loss, metabolic rates and blood chemistry during long-term fasting in alligators

Introduction

Many reptiles, including alligators, are infrequent eaters and may go months without food. The gut of these reptiles is adapted to being inactive for long periods of time, to digest large meals and to spend days to weeks digesting the food. Snakes may fast one to two months even while active (Smith, 1976). The monitor lizard (*Varanus albigularis*) fasts for months during the dry season and gorges during the wet season when food is abundant (Phillips, 1991). Also, crocodiles survive for long periods without food due to the large amounts of stored energy dense fat (Garnett, 1985).

The effect of fasting on metabolic rates, blood variables and gastrointestinal morphology and physiology has been studied in several reptiles (Martin and Bagby, 1973; Gatten, 1980; Garnett, 1986; Secor and Diamond, 1995; Secor *et al.*, 1994; Secor and Phillips, 1997; Overgaard *et al.*, 2002; Starck *et al.*, 2002). Maintaining gut function is energetically expensive during digestion and down-regulation of the gut will aid in energy conservation during long term fasting. For example, snakes will down-regulate intestinal function and reduce intestinal mass significantly, enabling them to conserve energy between meals (Secor *et al.*, 1994; Secor and Diamond, 1995). In *Python molurus*, standard metabolic rates decreased from about 60 kJ/kg/day to about

30 kJ/kg/day after 6 weeks of fasting (Secor, 2001). Starck *et al.* (2002) found that caimans vary the masses of their small intestine, duodenum and liver depending on their nutritional state (feeding versus fasting), and concluded that caimans exhibit similar responses to fasting as had been found previously in snakes.

A P→S shunt reduces arterial blood oxygen levels and it has been suggested that shunting hypoxic blood to the tissues may induce a hypometabolic state in reptiles (Jackson, 1968; Hicks and Wang, 1996; Hochachka *et al.*, 1996; Hicks and Wang, 1999). For example, Hicks and Wang (1999) showed that hypoxia resulted in a decrease in O₂ uptake in anesthetized turtles and concluded that a R→L shunt may be used as a strategy to prolong diving time in turtles by inducing a hypometabolic state of the tissues and thus reducing total body oxygen consumption. RV blood is also relatively acidic and shunting blood high in CO₂ may also be a strategy to induce a hypometabolic state. It has been suggested that the relative acidosis experienced by hibernating marmots might inhibit metabolic or ionic transport processes to promote the induction of hibernation (Malan *et al.*, 1973). Thus, shunting RV blood to the gut in alligators may aid in energy conservation by inducing a hypometabolic state in the gut tissues during long periods of fasting.

Blood levels of glucose, ketone bodies and creatinine can be used as indicators of the fasting phase of an animal. Glucose levels in the blood usually decrease initially and then reach a plateau during fasting. At the same time blood ketone bodies rise due to increased synthesis from lipids. Creatinine levels, an indicator of protein catabolism, increase initially and plateau after protein sparing sets in.

In this study we investigated the effects of long-term fasting in the two groups of alligators on rate of mass loss, whole body metabolic rates and blood glucose, total protein and creatinine. We hypothesized that the ability to shunt RV blood to the body during fasting would induce a hypometabolic state in the tissues and thus aid in energy conservation during long-term fasting. We predicted that during long-term fasting alligators with their LAo intact would lose mass at a slower rate than alligators with their LAo cut. We also predicted that metabolic rates would be higher in alligators with their LAo cut due to the inability to rapidly induce a hypometabolic state in the tissues. Blood glucose, total protein and creatinine levels were expected to be higher in alligators with their LAo cut, indicative of increased protein catabolism due to a higher metabolic rate. We fasted our alligators for 38 weeks in order to evaluate changes during phase I (glycogen as the primary fuel) and phase II (lipid stores as the primary fuel) of long-term fasting.

Material and Methods

Mass changes during fasting

Mass (kg; ± 0.001 kg) and length of alligator from the tip of the snout to the tip of the tail (cm) were recorded weekly for 38 weeks.

Blood sampling and analyses

Please see Chapter 2 for details on blood sampling and analysis. We measured whole venous blood variables just before and during fasting to determine the fasting

stages of the two groups of alligators. Blood was taken one week before fasting started, at the first day of fasting and every three weeks after, with the exception of the last sample which was delayed one week (four week interval). These intervals were chosen due to constraints at the laboratory where blood was analyzed, and to minimize the stress level on the alligators.

Blood (5 ml) was taken from the cervical sinus as described in Chapter 2. A 1 ml sub-sample of that blood was immediately injected into the pH blood/gas analyzer for measurements of pH, PCO₂ and PO₂ (see Chapter 2 for detailed description and Chapter 4 for results) and 4 ml was put into Serum Separation tubes (SST; Becton Dickinson, USA) for the analysis of serum variables and immediately put on ice. The SST tubes were centrifuged at 3000 rpm for 10 minutes within 30 minutes of blood withdrawal. The serum was aliquoted with a 1 ml syringe and stored in cryo-safe tubes on ice. The serum samples were immediately taken to St. Paul's Hospital (Vancouver, BC) for analysis of serum glucose, creatinine and protein levels.

Oxygen consumption and carbon dioxide release

The experimental set-up used to record rates of $\dot{V}O_2$ and $\dot{V}CO_2$ was the same as described in Chapter 2. Measurements were taken at weeks 25 to 38 after the onset of fasting. A mass spectrometer (QP 9000; Morgan Medical Ltd.) was used to measure O₂ and CO₂ concentrations. The mass spectrometer was calibrated with a standardized gas mixture (Praxair, Vancouver, BC). A subsample of the excurrent air leaving the funnel was drawn through a drierite column (drying agent) upstream of the mass spectrometer.

O₂ and CO₂ concentrations were recorded on a computer using a data acquisition program (Labtech, USA). On the recording, downward deflections from the baseline (ambient O₂ concentration) represented changes in O₂ concentration and upward deflections from the baseline (ambient CO₂ concentration) represented changes in CO₂ concentration. The respirometry system was checked for stability and leaks by injecting known quantities of nitrogen into the funnel after each experiment.

Data analysis and statistics

Statistical analyses were performed using JMP (SAS Institute, Inc.) and SigmaStat software (Jandel Corporation, USA). Unpaired t-tests were used to test for significant differences between groups. To test for the effects of mass, treatment (LAo cut) and nutritional state (fasting versus feeding) on variables, an Analysis of Covariance (ANCOVA) was used. Significance was accepted at the level of $P < 0.05$. All values are presented as Mean $\pm$ SEM.

Mass changes

Mass changes during fasting were recorded as % change in mass per group per week and averaged over 4-week periods to minimize noise in the data. We also calculated the total mass loss of each group of alligators (LAo intact and LAo cut) during the whole fasting period. This was recorded as a % of mass lost during the whole fasting period per group $((\text{Final mass (kg)} - \text{Initial mass (kg)}) * 100) / \text{Initial mass (kg)}$.

Blood chemistry

Serum blood glucose, creatinine and total protein values were averaged weekly per group of alligators.

Oxygen consumption and carbon dioxide release

$\dot{V}O_2$ and $\dot{V}CO_2$ were recorded for 7.5 ± 0.2 hours (mean $\pm$ SEM). Data were averaged over the entire period, except for the first hour. $\dot{V}O_2$ and $\dot{V}CO_2$ were determined using a data analysis program (Acqknowledge, BIOPAC Systems, Inc.). All values were corrected to STPD.

Results

Mass changes

There were no significant differences in mass measurements between the two groups of alligators before and during the whole fasting period. The average mass at the beginning of the fasting period was 4.01 ± 0.65 kg (Mean $\pm$ SEM) and 4.38 ± 0.56 kg for alligators with their LAo intact and LAo cut, respectively (Table 3.1). At the end of the 38 week fasting period the average mass was 2.9 ± 0.51 kg and 3.34 ± 0.44 kg for alligators with their LAo intact and LAo cut, respectively. The total amount of mass lost in each group was 27.4 ± 0.9 % for alligators with their LAo intact and 23.9 ± 1.6 % for alligators with their LAo cut (Table 3.1). Average weekly mass measurements during the 38 week fasting period are shown in Figure 3.1. Both groups of alligators lost mass

more rapidly during the first 12 weeks of the fasting period (Figure 3.2). At week 13 the rate of mass-loss stabilized at around -0.5 % per group per week.

Blood chemistry

There were no significant differences between the two groups of alligators in serum glucose, creatinine and total protein levels during the fasting period. Serum glucose levels decreased during fasting to around 5 mmol/L at week 9 in both groups of alligators from pre-fasting levels of about 7.5 (Lao intact) to 8.0 (LAo cut) mmol/L (Figure 3.3). After 9 weeks of fasting, serum glucose levels were significantly lower from average feeding values for both groups of alligators and remained significantly lower until the end of fasting. Serum creatinine levels at the beginning of the fasting period were significantly higher than feeding values (from about 38 μ moles/L to about 60 μ moles/L). Creatinine values stayed significantly elevated during the fasting period for both groups of alligators (except for week 15). Total protein levels in both groups of alligators were not significantly different from feeding levels.

Oxygen consumption and carbon dioxide release

The average mass when $\dot{V}O_2$ and $\dot{V}CO_2$ were recorded was 3.36 ± 0.48 kg and 3.64 ± 0.33 kg for alligators with their LAo intact and alligators with their LAo cut, respectively. There was no significant difference in average mass during the recording period between the two groups of alligators.

The $\dot{V}O_2$, $\dot{V}CO_2$ and RER values were not significantly different between the two groups of alligators during fasting (Figure 3.4; $\dot{V}O_2$: $P = 0.505$; $\dot{V}CO_2$: $P = 0.590$; RER: $P = 0.954$). During fasting, mass had a significant effect on $\dot{V}CO_2$ values ($F = 22.04$, $P = 0.0005$), but there was no significant difference between the two groups of alligators. Average $\dot{V}O_2$ values were 0.050 ± 0.006 ml/g/hr and 0.054 ± 0.006 ml/g/hr for alligators with their LAo intact and alligators with their LAo cut, respectively (Table 3.2). Average $\dot{V}CO_2$ values were 0.0127 ± 0.001 ml/g/hr and 0.0134 ± 0.0008 ml/g/hr for alligators with their LAo intact and alligators with their LAo cut, respectively. Both groups had low RER values, 0.261 ± 0.022 for alligators with their LAo intact and 0.263 ± 0.021 for alligators with their LAo cut.

Effect of nutritional states on measured respiratory variables

We also evaluated if nutritional state, i.e. feeding versus fasting, had an effect on the respiratory variables (Figures 3.5, 3.6; Table 3.2). Nutritional state did affect $\dot{V}O_2$ and RER values significantly ($\dot{V}O_2$: $F = 5.9$, $P = 0.024$; RER: $F = 41.44$, $P < 0.0001$). $\dot{V}O_2$ scaled positively with an increase in mass ($\dot{V}O_2$ (ml/hr) = $0.032 * g + 45.89$; Figure 3.5), as did $\dot{V}CO_2$ ($\dot{V}CO_2$ (ml/hr) = $0.015 * g - 0.013$). Furthermore, $\dot{V}CO_2$ was significantly affected by the amount of food eaten during feeding (food intake: $F = 17.6$, $P = 0.0004$; mass: $F = 56.0$, $P < 0.0001$). During feeding, average mass-specific $\dot{V}CO_2$ and RER values were significantly higher in alligators with their LAo cut compared with fasting values (Table 3.2). In alligators with their LAo intact, RER values were

significantly higher during feeding. Average $\dot{V}O_2$ values were lower during feeding compared with fasting for both groups of alligators, but this difference was not significant. Figure 3.6 shows the $\dot{V}O_2$, $\dot{V}CO_2$ and RER values within each group of alligators during feeding and fasting. The $\dot{V}O_2$ values were not significantly different during feeding compared with fasting for both groups of alligators ($F = 1.1$; $P = 0.3$). In alligators with their LAO, intact feeding $\dot{V}O_2$ values were higher but this was due to the effect of mass and not the nutritional state. The $\dot{V}CO_2$ values were significantly higher in both groups of alligators during feeding from fasting values ($F = 20.48$; $P = 0.0001$), as were RER values ($F = 41.06$; $P = 0.0001$).

Discussion

Both groups of alligators lost mass at similar rates during fasting and we found no difference in any of the blood or respiratory variables measured between groups. Thus the metabolic state of both groups was similar, suggesting that similar types (i.e. glycogen, fat or protein) and amounts of energy stores were metabolized. Measured blood variables showed that both groups of alligators were at similar fasting states throughout the 38 week fasting period.

An interesting finding is that it took 9 weeks for glucose levels to reach a plateau, whereas in mammals the plateau is usually reached after several hours (during the post-absorptive stage). This might indicate that alligators were still digesting food up to 9 weeks after fasting or that glycogen stores are large in these animals. In rattlesnakes average blood glucose levels were at 5.56 mmol/L (4.7 mmol/L to 6.4mmol/L) and total

protein levels were at 43 g/L (29 g/L to 56 g/L) after 20 weeks of fasting (Martin and Bagby, 1973). Our alligators exhibited a very similar response in blood glucose and protein levels during long-term fasting.

The expected RER values during the later stages of fasting would be around 0.7 (fat catabolism), but average RER values were 0.261 and 0.263 for alligators with their LAo intact and LAo cut, respectively. Why were these RER values so “low”? As discussed in Chapter 2 of this thesis, “typical” RER values are not necessarily seen in reptiles due to retention of CO₂ at the tissues or excretion of CO₂ at other sites such as kidneys and feces (Hall, 1924; Potter and Glass, 1931; Cook, 1949; Smith, 1975a; Grigg, 1978; Coulson and Hernandez, 1983; Atkinson and Bourke, 1984; Stinner *et al.*, 1996). Kleiber (1975) addressed the issue of unusually low RER values, i.e. below 0.7. He mentioned that RER values below 0.7 could be a result of the synthesis of carbohydrates during fasting and that theoretical RER values for gluconeogenesis from fat may be from 0.65 to 0, depending on the number of beta-hydroxy-butyric acid molecules (ketone bodies) produced during starvation. During fasting, triacylglycerides are hydrolyzed into glycerol and fatty acids. The glycerol is then converted to glyceraldehyde-3- P which is processed by glycolysis or gluconeogenesis. The fatty acids are oxidized to acetyl-CoA which is used to synthesize ketone bodies. We did not measure ketone bodies in our alligators but it can be assumed that after 25 weeks of fasting our alligators were breaking down fat stores to use as an energy source because crocodiles primarily lose fat stores during fasting (Garnett, 1986) and our alligators lost about 27 % and 24 % of body

mass. Similarly, hatchling *Crocodylus porosus* lost 22.7 % of mass after 58 days without food and lost 34.1 % of their endogenous protein and 50 % of fat.

Our low RER values could have been a result of non-pulmonary CO_2 loss by the kidneys as NH_4HCO_3 . Even though nitrogen excretion should have been low in our alligators due to the lack of protein intake, it could have been somewhat elevated during the time of recording. At 25 to 38 weeks of fasting, the time period when we recorded our respiratory variables, protein catabolism was elevated as was seen in the significantly higher creatinine levels. At early times of starvation, hepatic glycogenolysis is the major source of blood glucose, leading to glycogen depletion. Catabolism of amino acids is characteristic of the later stages of starvation and gluconeogenesis from amino acids, glycerol and lactate becomes the source of blood glucose. During the early stage of starvation, nitrogen is primarily excreted as urea. The major site of gluconeogenesis is initially the liver, but as starvation progresses, there is a slow decrease in the amount of urea excreted and an increase in the amount of ammonia excreted, coinciding with the increased rate of kidney gluconeogenesis. This change in the site of gluconeogenesis from amino acids is a consequence of the role of the kidney in acid-base balance. During starvation, metabolic acidosis becomes a problem due to the increased synthesis of ketone bodies and the kidneys have to increase the rate of proton excretion. Proton excretion in the kidneys is buffered by increased ammonia production from glutamine as carbon atoms are converted to glucose in the kidneys. Alligators primarily excrete their nitrogenous wastes as ammonium bicarbonate (NH_4HCO_3) when hydrated and our alligators always had access to water. At the later stages of fasting nitrogen excretion

was probably elevated due to the increased role of the kidney in gluconeogenesis and due to the increased protein catabolism inferred from creatinine levels observed at weeks 25 to 38 (Figure 3.3). When fasting respiratory variables were recorded, creatinine levels were about 89 % and 76 % above average feeding levels in alligators with their LAo intact and LAo cut, respectively. Unfortunately, we did not measure urine and feces variables at that time. Based on the $\dot{V}O_2$ values during fasting, the calculated amount of CO_2 from nitrogen excretion would be 1.2 L/day and 1.4 L/day for alligators with their LAo intact and LAo cut, respectively (see Chapter 2 for calculation details adapted from Grigg, 1978). Assuming a RER of 0.7 ("normal" fasting RER), $\dot{V}CO_2$ would have been 2.8 L/day and 3.3 L/day for alligators with their LAo intact and LAo cut, respectively. So, calculated RER values would be 0.41 for both groups of alligators ($(2.8 \text{ L/day } CO_2 - 1.2 \text{ L/day } CO_2)/4.0 \text{ L/day } O_2$ and $(3.3 \text{ L/day } CO_2 - 1.4 \text{ L/day } CO_2)/4.7 \text{ L/day } O_2$) for alligators with their LAo intact and LAo cut, respectively). Hence, nitrogen excretion alone does not explain the low RER values in our alligators.

Retention of CO_2 at the tissues may also be a strategy to induce a hypometabolic state. In hibernators mechanisms in addition to hypothermia, such as decreases in intracellular pH, are thought to contribute to metabolic arrest (Malan, 1978; 1988). Recent studies in the peanut worm have also shown that a decrease in extracellular pH from control levels (7.9 to 6.7) caused a reduction in metabolic rate (Langenbuch and Poertner, 2002). Thus, the combination of CO_2 excreted as NH_4HCO_3 , the increased amount of ammonia excretion during the later phases of starvation and CO_2 retention at

the tissues may contribute to the low $\dot{V}CO_2$ values in our alligators and thus low RER values.

We evaluated if nutritional state had an effect on respiratory variables. RER and $\dot{V}CO_2$ values were significantly higher during feeding in each group compared with fasting values (Table 3.2). The GI tract is highly aerobic during feeding and digestion and growth require a high level of metabolic processes. Tissue respiration increases during feeding and this is usually reflected in higher $\dot{V}O_2$ and $\dot{V}CO_2$ values. Secor and Diamond (1997) found that protein synthesis following feeding may contribute 20-25% to the metabolic rate in a python. Garnett (1986) found average $\dot{V}O_2$ values of 0.041 ml/g/hr (0.036 ml/g/hr to 0.055 ml/g/hr) and average $\dot{V}CO_2$ values of 0.030 ml/g/hr (0.023 ml/g/hr to 0.042 ml/g/hr) in hatchling crocodiles with an average mass of 358 g. These crocodiles had been fasted until feces production ceased (more than 5 days). Our $\dot{V}O_2$ values were similar to the values found by Garnett (1986) and our alligators had been fasting for 25 weeks before measurements were made. These findings indicate that the length of fasting did not affect $\dot{V}O_2$ values in our alligators. One surprising result was that average feeding $\dot{V}O_2$ values were lower than average fasting $\dot{V}O_2$ values in our alligators. The higher fasting $\dot{V}O_2$ values were due to the effect of size on $\dot{V}O_2$ values (Figure 3.4). Alligators with their LAo intact were significantly larger during feeding ($P = 0.001$). This was not the case for alligators with their LAo cut ($P = 0.641$). Also, protein catabolism increases $\dot{V}O_2$ in animals and as noted above, protein catabolism was elevated during the time of recording respiratory variables in both groups of alligators.

An interesting finding was the changes within each group (Figure 3.6). In alligators with their LAo intact, the RER value of 0.3 during feeding was from one alligator that had not eaten for 13 days prior to recording feeding metabolic rates. Essentially this alligator had been fasting, and its RER value of 0.3 was similar to RER values found during the fasting experiment.

A P→S shunt affects arterial blood gases and pH. In turtles, R→L shunting during diving is thought to induce a hypometabolic state of the tissues and thus prolong dive time (Jackson, 1968; Hicks and Wang, 1999). For example, Hicks and Wang (1999) found that a reduction in arterial oxygen levels in anaesthetized turtles resulted in a 30% reduction in $\dot{V}O_2$. A reduced arterial pH has also been shown to reduce $\dot{V}O_2$ in isolated canine hindlimbs (Harken, 1976), and Malan *et al.* (1973) suggested that the relative acidosis experienced in hibernating marmots may inhibit metabolic or ionic transport processes. Alligators can affect their arterial blood gases and pH by changes in ventilation and via the P→S shunt. We found no differences in measured respiratory variables during fasting between the two groups of alligators, nor in any of the other measured variables such as mass loss. If adjustments were made in ventilation to affect arterial blood gas levels and pH in order to induce a hypometabolic state in tissues, they were similar in both groups of alligators and ligating the LAo did not affect this ability. Thus, these results show that the ability to shunt RV blood to the digestive system is of no benefit during fasting. $\dot{V}CO_2$ and RER values were lower during fasting but $\dot{V}O_2$ were higher. However, that was due to the effect of mass on $\dot{V}O_2$ and due to the elevated protein catabolism during fasting when respiratory variables were recorded.

We found that alligators with their LAo cut experienced larger changes in $\dot{V}CO_2$ and RER values during feeding compared with fasting. $\dot{V}CO_2$ was 161% higher and RER values were 242% higher during feeding in alligators with their LAo cut (Figures 3.5, 3.6 and Table 3.2). In alligators with their LAo intact these changes were only 115% and 151% for $\dot{V}CO_2$ and RER values, respectively. Animals regulate their acid-base status via the respiratory system and kidneys. However, alligators can impact their acid-base status by shunting RV blood to the body. During feeding alligators experience acid-base disturbances due to food digestion and shunting RV blood to the body may aid in buffering these acid-base disturbances (i.e. the alkaline tide). Alligators with the ability to shunt RV blood to the body can alter arterial PO_2 , PCO_2 levels and pH via adjustments in ventilation and via the shunt. Alligators with their LAo cut can not use the shunt but can adjust their ventilation in response to acid-base disturbances and this is may be reflected by $\dot{V}CO_2$ and RER values during feeding.

Figure 3.1. Weekly average mass measurements (kg) in two groups of alligators during long-term fasting. LAo intact: N=4; LAo cut: N=6. All values are Means $\pm$ SEM. For clarity, only positive SEM's are shown for alligators with their LAo cut and for alligators with their LAo intact only negative SEM's are shown.

Figure 3.2. Average rate of mass-loss (% per group per week) during long-term fasting in two groups of alligators. LAo intact: N=4; LAo cut: N=6. All values are Means $\pm$ SEM.

Figure 3.3. Serum blood levels before and during long-term fasting in two groups of alligators. The fasting period started at time "0". Red circles denote feeding values just prior to fasting. A - Serum glucose levels (millimoles per liter); B - Serum creatinine levels (micromoles per liter); C - Serum total protein levels (grams per liter). LAo intact: N=4; LAo cut: N=6. Values are shown as means $\pm$ SEM. Significant differences from feeding values are indicated by an asterisk.

Figure 3.4. Respiratory variables during fasting in two groups of alligators. $\dot{V}O_2$, rate of oxygen consumption; $\dot{V}CO_2$, rate of carbon dioxide release; RER, respiratory exchange ratio; LAo intact: N=4; LAo cut: N=6.

Figure 3.5. Respiratory variables during feeding and fasting in two groups of alligators. $\dot{V}O_2$, rate of oxygen consumption; $\dot{V}CO_2$, rate of carbon dioxide release; RER, respiratory exchange ratio; Red circles denote values recorded 6 days after feeding and blue circles denote values recorded 25 to 38 weeks during fasting. LAo intact: N=4; LAo cut: N=6.

Figure 3.6. Respiratory variables during feeding and fasting in two groups of alligators. $\dot{V}O_2$, rate of oxygen consumption; $\dot{V}CO_2$, rate of carbon dioxide release; RER, respiratory exchange ratio; Red circles denote values recorded 6 days after feeding and blue circles denote values recorded 25 to 38 weeks during fasting. LAo intact: N=4; LAo cut: N=6. The horizontal line in each graph represents the mean for each group.

Table 3.1. Average mass measurements during long-term fasting in two groups of alligators.

	Initial mass (kg)	Final mass (kg)	Total mass lost (%)
LAo intact	4.01 ± 0.65	2.9 ± 0.51	27.44 ± 0.89
LAo cut	4.38 ± 0.56	3.34 ± 0.44	23.98 ± 1.56

Legend:

Total mass lost: $(\text{Initial mass} - \text{final mass}) * 100 / \text{Initial mass}$

LAo intact during fasting: N=4; LAo cut during fasting: N=6.

Table 3.2. Average $\dot{V}O_2$, $\dot{V}CO_2$ and RER values during fasting and feeding in two groups of alligators.

	$\dot{V}O_2$ (ml/g/hr)	$\dot{V}CO_2$ (ml/g/hr)	RER
<u>Fasting:</u>			
LAo intact	0.050 ± 0.006	0.013 ± 0.001	0.261 ± 0.022
LAo cut	0.054 ± 0.006	0.013 ± 0.001	0.263 ± 0.021
<u>Feeding:</u>			
LAo intact	0.038 ± 0.003	0.015 ± 0.001	$0.395 \pm 0.037^\dagger$
LAo cut	0.032 ± 0.003	$0.021 \pm 0.002^{\dagger*}$	$0.638 \pm 0.019^{\dagger*}$

Legend:

$\dot{V}O_2$: oxygen consumption rates; $\dot{V}CO_2$: carbon dioxide release; RER: respiratory exchange ratio.

LAo intact during feeding: N=3; LAo cut during feeding: N=3.

LAo intact during fasting: N=4; LAo cut during fasting: N=6.

Significant differences between groups are indicated with an asterisk and significant differences between fasting and feeding within each group are indicated with a cross.

CHAPTER 4

The acid-base status of two groups of alligators

Introduction

Many crocodilians ingest large meals at infrequent intervals, leading to significant changes in acid-base status due to the secretion of large amounts of hydrochloric acid (HCl) into the stomach. This results in a metabolic alkalosis known as the alkaline tide and is reported to be very large in alligators, with pH values above 8.0 and a large decrease in chloride (Cl^-). The large increases in bicarbonate (HCO_3^-) values after feeding were a result of the secretion of large amounts of HCl (Figure 4.1; Coulson *et al.*, 1950). A more recent study (Busk *et al.*, 2000b) reported much smaller acid-base changes in alligators after feeding: fasting pH was 7.51 and increased to 7.6 24 hours after feeding. The pH values measured at 24 hours after feeding were similar in both studies (Coulson *et al.*, 1950; Busk *et al.*, 2000b) but fasting values were different. They were lower in the study by Coulson and colleagues (1950). In the study by Busk *et al.* (2000b), alligators had chronically implanted catheters to minimize stress during blood sampling but Coulson and colleagues (1950) obtained blood by cardiac puncture, which can involve higher levels of stress in the animals and thus affect measured pH as well as PCO_2 values (also discussed in this study). Even though arterial pH did not change significantly, HCO_3^- and PCO_2 values increased significantly in alligators following feeding in the study by Busk *et al.* (2000b). The significant increase in PCO_2 by a respiratory compensation during the postprandial period maintained pH values around

fasting levels in their study. Thus, in both studies feeding resulted in significant changes in acid-base status in alligators.

Figure 4.1. A - Average change in plasma pH and B - average change in plasma chloride (open squares) and plasma bicarbonate (closed squares) in alligators after feeding (after Coulson *et al.*, 1950).

In the present study the effect of ligating the LAo on the acid-base status following feeding was investigated. The hypothesis was that the ability to shunt RV blood to the body aids in reducing the alkaline tide during feeding. We predicted that alligators with their LAo cut would experience larger and/or longer acid-base disturbances following feeding, including a larger alkaline tide and whole blood PCO₂ values should be lower in alligators with their LAo cut due to the inability to shunt RV blood to the body. Also, if alligators employ the shunt in response to feeding in order to

ameliorate the alkaline tide, acid-base status (changes in PCO_2 , pH and HCO_3^-) should be similar in both groups of alligators during long-term fasting.

Temperature has significant effects on respiratory and acid-base balance in reptiles (for reviews see Reeves, 1969; Rahn, 1974; Howell and Rahn, 1976; Reeves, 1977; Heisler, 1986; Jackson, 1986). Reptiles don't maintain a constant pH and PCO_2 with changing body temperature. The general picture is that as temperature increases, pH decreases and PCO_2 increases. Rahn (1967) proposed that plasma pH in poikilothermic vertebrates is regulated as body temperature changes so as to maintain a constant relative alkalinity, i.e. a constant $[\text{OH}^-]/[\text{H}^+]$ ratio. Reeves (1972) suggested that PCO_2 is regulated by way of ventilation so that the fractional dissociation (α , α) of the imidazole moiety of histidine is kept constant (imidazole alphastat hypothesis). As the pK' of imidazole changes with body temperature in about the same manner as the neutral pH of water, the alphastat hypothesis is consistent with that of constant relative alkalinity (Heisler, 1986). Reeves (1972) defined α which is the fraction of total titratable imidazole groups that were ionized by: $\alpha = 10^{(\text{pH}-\text{pK})}/(1 + 10^{(\text{pH}-\text{pK})})$. So, if the change in pK with temperature is the same as the change in pH with temperature, α remains constant and the change in pK with a change in body temperature ($\Delta\text{pK}/\Delta T$) of imidazole ranges from -0.019 to -0.016 U°C (Heisler, 1986). The acid-base adjustments associated with changes in body temperature are regulated via a combination of respiratory and metabolic (ionic) mechanisms adjusting PCO_2 to the appropriate pH to maintain protein charge in ectotherms. The kidneys play an important role in acid-base

balance during chronic acid-base disturbances, but it has been shown that they play a minor role following acid-base disturbances in reptiles (Glass and Heisler, 1986; Silver and Jackson, 1986). Reptiles primarily employ their respiratory system to rapidly adjust to acid-base disturbances. Increasing arterial PCO_2 levels via adjustments in ventilation brings about a decrease in blood pH values with an increase in body temperature. In crocodilians, PCO_2 can also be increased via a P→S shunt (Weber and White, 1986).

It has been proposed that shunting may also play a role in thermoregulation in reptiles (Burggren, 1987). A net R→L shunt may facilitate warming by perfusing the periphery with relatively cooler blood during basking. On the other hand, a L→R shunt would facilitate cooling by recirculating warmer blood through the lungs. For example, Morgareidge and White (1969) showed that cutaneous blood flow during warming increased in the marine iguana. In alligators, cutaneous blood flow is reduced during cooling and increased during warming (Smith, 1975a).

Alligators have a preferred body temperature of 31° C and digestion and absorption of nutrients are slow and incomplete at temperatures lower than 25° C (Coulson and Coulson, 1986; Coulson *et al.*, 1990). Alligators were exposed for 16 weeks to 20° C, although most studies have only looked at acid-base changes in response to temperature changes for hours or days (Austin *et al.*, 1927; Davies, 1978; Davies *et al.*, 1982; Lutz *et al.*, 1989; Douse and Mitchell, 1991). Our alligators were kept at 20° C for many weeks to ensure that no additional time dependent changes in acid-base status would occur as a result of metabolic changes or strong ion adjustments. We stopped feeding our alligators during the 20° C experiments (week 9 at 20° C). This allowed us to

evaluate the effect of temperature alone on acid-base status in both groups of alligators and eliminated the combined effects of temperature and feeding (alkaline tide) on acid-base status. We predicted that alligators with their LAo cut would show a more alkaline pH during feeding at the lower temperature and that lack of food intake should reduce whole body pH even at the lower temperature. At the lower temperature, digestion rates and rates of HCl secretion are reduced. For example, Coulson *et al.* (1990) showed that rates of protein digestion were reduced when alligators were exposed to 20° C from 31° C. Alligators with their LAo intact can shunt RV blood to the gut to help in ameliorating the alkaline tide. Thus, we predicted a more alkaline pH in alligators with the LAo cut at the lower temperature while digesting food due to the inability to shunt RV to the body.

Material and Methods

Animal care and maintenance

Details regarding surgical procedures, the holding facilities and animal care of the two groups of alligators are given in Chapter 2. All surgical procedures and experiments were approved by the University of British Columbia Committee on Animal Care.

Whole blood pH, PO₂ and PCO₂

The procedure for capturing the alligator, taping the mouth and taking blood was timed using a stopwatch. The time to take blood was recorded because blood measurements such as PCO₂, PO₂ and pH are affected by this time factor. Blood was

taken from the cervical sinus into a 5 ml syringe using a sterile 1.5 inch, 20 gauge needle. Immediately following blood sampling, whole blood pH and gases (PCO_2 and PO_2 , mmHg) were measured using a pH/blood gas analyzer (Instrumentation Laboratory, Inc.). The analyzer was calibrated using standard pH (7.384 and 6.840) and gas mixtures (21.08% O_2 , 5.03% CO_2 , balance Nitrogen and 0% O_2 , 10.2% CO_2 and balance Nitrogen) at 30° C which was the average water temperature in the alligator rooms and usually the average body temperature of the alligators measured via a rectal thermometer. The analyzer was recalibrated every 20 minutes during the blood taking procedure. The process of taking blood from all the alligators lasted for about 1 hour. Hematocrit was measured using hematocrit tubes. Immediately following gas analysis and hematocrit measurements, 3 ml of blood were put into plasma tubes (Becton Dickinson, USA) for the analysis of plasma variables and immediately iced. Plasma tubes were centrifuged at 3000 rpm for 10 minutes within 30 minutes of blood sampling. The plasma was aliquoted with a 1 ml syringe and stored -8° C until further analysis.

Changes of whole venous blood pH and PCO_2 during fasting

Whole venous blood pH and PCO_2 (mmHg) were measured during long-term fasting. Blood was taken 6 weeks, 9 weeks and 18 weeks after the last feeding and every three weeks after that with the last two samples taken at four-week intervals.

Blood variables during feeding

Whole venous blood pH, PCO₂ and PO₂ were measured just before feeding, 25 hours, 72 hours and 121 hours after feeding. These intervals were based on previous studies on blood pH and gas measurements made in alligators following feeding (Coulson *et al.*, 1950). All alligators were weighed before feeding and 25 hours following feeding to determine food intake (including water intake; see Chapter 2).

Plasma chloride levels were measured using a digital chloridometer (Haake Buchler Instruments, Inc.). 10 microliters of plasma per alligator were used to determine chloride values as milliequivalents per liter (mEq/l).

Temperature changes

Whole venous blood pH and gases were analyzed in the two groups of alligators at 30° C and 20° C during feeding and fasting. One sample of blood was taken at 30° C just before the water and room temperature were changed to 20° C. During the 20° C measurements, the blood pH/gas analyzer was calibrated at 20° C. Alligators were fed whole chicken pieces, rats or mice *ad lib* just after blood measurements were made. The alligators were not fed after week 9, so food intake for measurements at week 11, 13 and 16 was zero for both groups.

Blood was taken at 1, 3, 5, 9, 11, 13 and 16 weeks exposure to 20° C. Temperature was then increased to 30° C and blood samples were taken at 1, 3, 5 and 9 weeks. During this group of experiments at 30° C, food intake per group of alligators

was measured exactly because the two groups were separated during feeding. Food intake was measured as a % of total body mass of food intake per group per week.

Body temperature was measured using a thermocouple digital display meter (Model 8700, Mallinckrodt Inc., Glens Falls, NY) and a 2 mm diameter rubber coated thermocouple probe. The probe was gently inserted to approximately 7-10 cm into the cloaca and readings were taken once the display reached a stable value.

Data analysis and statistics

Statistical analyses were performed using JMP (SAS Institute, Inc.) and SigmaStat software (Jandel Corporation, USA). Unpaired t-tests were used to test for significant differences between groups. To test for the effects of treatment (LAo cut), food intake, body temperature and time to take the blood, an Analysis of Covariance (ANCOVA) was used. Significance was accepted at the level of $P < 0.05$. All values are presented as mean $\pm$ SEM.

Changes in whole venous blood pH and PCO₂ during fasting

During fasting, pH and PCO₂ values were averaged weekly per group of alligators.

Blood variables during feeding

During feeding, pH, PCO₂ and PO₂ values were averaged per group of alligators. ANCOVA was used to test for significant effects of treatment (ligating the LAo) or food

intake (% of total body mass of food intake per alligator, including water intake) on pH, PCO₂ and PO₂ changes after a meal.

Plasma bicarbonate (HCO₃⁻) values were calculated from measured PCO₂ and pH values using the Henderson-Hasselbalch equation at 30° C (see Jensen *et al.*, 1998 for details): $\text{pH} = \text{pK} + \log \text{HCO}_3^- / \text{PCO}_2 * \alpha\text{CO}_2$, where pH was measured, pK is the dissociation constant (calculated as $\text{pK} = -0.0817 * \text{pH} + 6.7818$); PCO₂ was measured and αCO_2 is the solubility constant and was 0.0366 mmol * l⁻¹ * mmHg⁻¹. Values were averaged per group of alligators.

Chloride values were averaged per group of alligators.

Temperature changes

In response to temperature changes, whole venous blood pH, PCO₂ and PO₂ values, the time it took to sample blood (seconds) and body temperatures were averaged per group of alligators.

Plasma HCO₃⁻ values were calculated from the measured PCO₂ and pH values using the Henderson-Hasselbalch equation at 30° C as described above.

Results

Changes of whole venous blood pH and PCO₂ during fasting

The average mass between the two groups of alligators was not significantly different during fasting (Chapter 3). Figure 4.2 shows whole venous blood pH and PCO₂ changes during long-term fasting. Average whole venous blood pH values were not

significantly different between the two groups of alligators. Average pH was significantly higher than starting values (week 6) after week 21 for alligators with their LAo intact and after week 34 for alligators with their LAo cut.

Average whole venous blood PCO₂ levels were significantly different between the two groups of alligators at weeks 6 and 9 after the start of fasting ($P < 0.003$ and $P < 0.037$, respectively). They were significantly lower in alligators with their LAo cut. This difference disappeared after 18 weeks of fasting. Average PCO₂ values were significantly higher compared with values at the beginning of the fasting period in alligators with their LAo cut at weeks 9, 18, 21, 34 and 38. In alligators with their LAo intact, average PCO₂ values were significantly lower compared with values at the beginning of the fasting period at weeks 24 and 30.

Blood variables during feeding

The average mass during blood sampling was 6.27 ± 0.96 kg and 4.22 ± 0.49 kg for alligators with their LAo intact and LAo cut, respectively. These were not significantly different. Average food intake during these experiments was 4.31 ± 0.83 % and 2.79 ± 0.42 % (% of total body mass per alligator, including water intake) for alligators with their LAo intact and LAo cut, respectively. This difference was not significant.

Changes of whole venous blood pH, PCO₂ and PO₂ values following feeding are shown in Figure 4.3. Average whole blood venous pH increased in both groups 25 hours after feeding. The pH increased from 7.44 ± 0.04 (mean $\pm$ SEM) and 7.48 ± 0.04 just

before feeding to 7.53 ± 0.05 and 7.60 ± 0.04 after 25 hours of feeding for alligators with their LAo intact and LAo cut, respectively. The increase in pH was not significant from pre-feeding values within each group of alligators and there was no significant difference between groups. There was also no significant effect of food intake on the increase in pH at 25 hours (LAo intact: $F = 1.87$, $P = 0.23$; LAo cut: $F = 2.05$, $P = 0.1$). After 72 hours pH values decreased in both groups of alligators and were similar to pre-feeding values by 121 hours after feeding.

Average PCO_2 values did not change significantly at 25 hours after feeding from pre-feeding values. In alligators with their LAo cut, average PCO_2 values were significantly lower from pre-feeding values at 72 hours ($P = 0.007$). At 72 hours average PCO_2 values were significantly different between the two groups of alligators: they were significantly lower in alligators with their LAo cut ($P = 0.05$). For both groups of alligators, average PCO_2 values were similar to pre-feeding values by 121 hours. Average PO_2 values did not change significantly following feeding in both groups of alligators and were not significantly different between the two groups of alligators.

Average HCO_3^- values and chloride (Cl^-) values following feeding are shown in figure 4.4. Average HCO_3^- and Cl^- values did not significantly change from pre-feeding values and there was no significant difference between the two groups of alligators.

Davenport diagrams are presented in Figure 4.5 for both groups of alligators.

Temperature changes

Figure 4.6 shows whole venous blood pH changes during 30° C and long-term exposure to 20° C. Average venous pH values were significantly higher at the initial 30° C measurement in alligators with their LAo cut. Average pH values were also significantly higher in alligators with their LAo cut at 1, 3, 5 and 16 weeks at 20° C exposure. After changing the temperature back to 30° C, pH values decreased in both groups of alligators. Values were significantly different between the two groups of alligators at 9 weeks at 30° C.

During the 20° C exposure, ligating the LAo had a significant effect on pH measurements ($F = 13.67$, $P = 0.0005$), as did the time to take blood ($F = 6.32$, $P = 0.01$). However, the average times to take blood during these experiments were not significantly different between the two groups of alligators as shown in Figure 4.7, except for week 16 at 20° C and average pH values were significantly higher in alligators with their LAo cut at 16 weeks at 20° C.

During 30° C, ligating the LAo had a significant effect on pH measurements ($F = 5.4$, $P = 0.025$), as did the time to obtain blood ($F = 6.15$, $P = 0.017$). Again, the average times to obtain blood were not significantly different between the two groups of alligators (Figure 4.7).

During 20° C exposure, average body temperatures were not significantly different between the two groups of alligators. During 30° C exposure, average body temperatures were significantly different at weeks 5 and 9. However, when testing for a

significant effect of temperature within the 30° C exposure, temperature did not have a significant effect ($F = 0.85$, $P = 0.36$).

Temperature significantly affected pH values ($F = 28.42$, $P < 0.001$). Average pH values were significantly higher at 20° C compared to values at 30° C for both groups of alligators. They were also significantly higher at week 3 and week 9 at 30° C after the 20° C exposure for alligators with their LAo intact. The average pH increased significantly from 7.40 ± 0.02 at 30° C to an average value of 7.64 ± 0.02 at 20° C in alligators with their LAo intact ($P < 0.001$). In alligators with their LAo cut, pH increased significantly from 7.55 ± 0.02 at 30° C to an average value of 7.80 ± 0.02 at 20° C ($P < 0.001$). Also, average pH values were significantly higher in alligators with their LAo cut compared to alligators with their LAo intact at both temperatures ($P < 0.001$).

The slope of $\Delta\text{pH}/\Delta^\circ\text{C}$ (change in pH per degree Celsius) for alligators with their LAo intact was $-0.0224 \text{ U}/^\circ\text{C}$ and for alligators with their LAo cut it was $-0.0307 \text{ U}/^\circ\text{C}$.

Average PCO_2 values were significantly higher in alligators with their LAo intact at the initial 30° C measurement and after 1 week at 20° C (Figure 4.6). Average values decreased significantly in both groups by week 3 at 20° C and stayed around 20 mmHg for both groups during the remaining 20° C exposure. After changing the temperature back to 30° C, average PCO_2 values increased significantly in both groups of alligators. In alligators with their LAo intact, average PCO_2 values were significantly lower than the initial 30° C measurement during weeks 1, 3, 5 and 9 at 30° C after the 20° C exposure. There was no significant difference between groups.

PO₂ values decreased significantly in both groups of alligators with a decrease in temperature (except for week 13) but there was no significant difference between the two groups of alligators. Average PO₂ values increased significantly after changing the temperature back to 30° C. These were not significantly different from the initial 30° C value for both groups of alligators.

The average calculated HCO₃⁻ values increased in both groups after 1 week at 20° C, then slightly decreased in both groups at week 3, increasing again at weeks 5, 9 and 11 (Figure 4.8). There was no significant difference between the two groups of alligators.

Figure 4.9 shows the average venous pH measurements during 30° C exposure when food intake was measured. Average whole venous blood pH values were higher in alligators with their LAo cut, even when food intake was lower.

Davenport diagrams are presented in Figure 4.10 for both groups of alligators at the two temperatures.

Discussion

We measured whole venous blood variables from the cervical sinus, which receives blood from the cephalic region. There are several reasons for this, which reflect disadvantages as well as advantages. Our alligators got very upset when handled, especially during blood taking. Our animals stopped their feeding and regurgitated their food when stressed. Taking blood from the cervical sinus is relatively quick and tends to minimize handling stress on the alligators opposed to taking blood via cardiac puncture.

Also, cannulating an arterial blood vessel was not possible. Our alligators attack anything that is not tied down and are very curious animals. In addition, isolating alligators from the group tends to really upset them and again they stop eating. Also, cannulating animals can affect their normal behavior as was found in turtles, which showed changes in normal diving behaviors after being cannulated (Lutz *et al.*, 1989).

Venous PO_2 and PCO_2 values reflect tissue metabolism. The oxygen content of venous blood will be determined by rates of O_2 delivery to the tissues and O_2 demand by the tissues. Anything that decreases oxygen affinity in the arterial blood (such as temperature, PCO_2) will result in higher venous PO_2 levels. An arterial sample is preferable to a venous sample to evaluate the respiratory system, i.e. gas exchange at the lungs. Bicarbonate is, however, very similar in arterial and venous blood. For example, if PCO_2 is decreased on the venous side, then it must be decreased on the arterial side, i.e. respiratory alkalosis. A central (jugular, pulmonary artery) venous PO_2 will give information on flow to the tissues and tissue oxygenation. A disadvantage of measuring mixed venous blood is that we do not know what the tissues were receiving in terms of arterial blood pH, PCO_2 and PO_2 .

The average whole venous blood pH was higher in alligators with their LAo cut during all of the experiments, especially when presented with a temperature challenge during feeding. Opposed to this trend was the lack of any significant differences in measured whole venous blood variables during long-term fasting, although average PCO_2 values were significantly lower at the beginning of the fasting period in alligators

with their LAo cut (Figure 4.2). These differences could have been due to a difference in digestive state between the two groups of alligators. Gastrosocopy revealed food in one alligator (LAo cut) 9 weeks after the onset of fasting but at the same time, an alligator with the LAo intact had an empty stomach. Unfortunately, we were not able to check for food in the stomachs of all the alligators in each group. Also, we did not measure food intake in these animals before the start of fasting and so cannot draw precise conclusions about these variables at the beginning of fasting. At 18 weeks after the start of fasting, there were no differences between the two groups of alligators in any of the variables measured.

Average pH values increased in both groups of alligators during long-term fasting from starting values during fasting. They increased significantly for alligators with their LAo intact after 21 weeks without food and after 34 weeks without food in alligators with their LAo cut. Also, PCO_2 values were significantly lower in alligators with their LAo cut at the beginning of the fasting period. Alligators with their LAo intact can increase their blood PCO_2 by altering their ventilation (hypoventilate; decrease $\dot{V}E/\dot{V}CO_2$) or via the shunt due to its relatively high CO_2 content. However, alligators with their LAo cut can only alter their ventilation to increase PCO_2 values. Whether or not alligators with their LAo intact employed their shunt at the beginning of the fasting period is not known. A reduction in blood PCO_2 during long-term fasting could be due to the cessation of the shunt if alligators employed the shunt during digestion. Once our alligators had been fasting for 18 weeks and were certainly post-absorptive, PCO_2 values were similar in both groups of alligators. So, if our alligators employed changes in

ventilation to alter their acid-base status, these changes were similar in the two groups during long-term fasting.

A more acidic pH, due to ketogenesis during fasting is expected. The two ketones, acetoacetate and β -hydroxybutyrate, are synthesized in the liver during starvation and released into the circulation. Both are organic acids and because they are released in the protonated form, tend to lower the pH. In our study, an increase in venous pH could be due to increased protein catabolism. Protein catabolism liberates HCO_3^- , which would result in a more alkaline pH. In the hydrolysis of protein, amino acids are produced and the oxidation of amino acids containing $-\text{COO}^-$ and $-\text{NH}_3$ groups result in HCO_3^- and ammonium (NH_4^+) production at about equal amounts. NH_4^+ can not donate a proton for the titration of HCO_3^- because it is too weak an acid (pK_a values are 6.1 for the $\text{CO}_2/\text{HCO}_3^-$ system and 9.25 for the $\text{NH}_4^+/\text{NH}_3$ system). In mammals, ureagenesis in the liver prevents a metabolic alkalosis via the urea cycle (Atkinson and Bourke, 1987). The overall reaction is as follows: $2 \text{HCO}_3^- + 2 \text{NH}_4^+ \leftrightarrow \text{H}_2\text{NCONH}_2$ (urea) + CO_2 + 3 H_2O . The urea cycle consumes four ATP and thus is energetically expensive. The urea diffuses into the blood which carries it to the kidneys where it is excreted. The CO_2 diffuses into the blood and is blown off at the lungs. Thus, in mammals, urea synthesis plays an important role in the regulation of pH. Alligators excrete their nitrogen waste as NH_4HCO_3 when hydrated and HCO_3^- is formed in the kidneys in the presence of carbonic anhydrase (CA; $\text{H}_2\text{O} + \text{CO}_2 \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{H}^+ + \text{HCO}_3^-$; Coulson and Hernandez, 1983). For example, if CA is inhibited, renal HCO_3^- synthesis is reduced and alligators will excrete their nitrogenous waste as NH_4Cl (Coulson and Hernandez, 1954). Because

HCO_3^- from protein catabolism can not directly be used to excrete NH_4HCO_3 in the kidneys, plasma pH in our alligators could have been elevated during long-term fasting due to the elevated protein catabolism. We measured significantly higher creatinine levels throughout the fasting period, indicating elevated protein catabolism. As we did not find any significant differences in acid-base status between the two groups of alligators during long-term fasting, the ability to shunt RV blood to the body was of no noticeable benefit.

Both groups showed small increases in pH after feeding (Figure 4.3). These changes were not significantly different between the two groups of alligators and were not as large as Coulson and his colleagues found (1950). Struggling during blood sampling can have large effects on blood pH and gas measurements. We measured the amount of time it took per alligator to sample blood. This allowed us to determine the effect of this time factor when comparing among and between measured variables in the two groups of alligators. This time factor was very important and did have significant effects on blood pH and gas measurements (more acidic pH and higher PCO_2 values with more struggling), so minimizing stress is a key when measuring blood pH and gases. Heisler (1986) pointed out that errors in blood measurements can be due to the "grab and stab" method during blood sampling because this method can result in shifts in plasma pH due to elevated stress levels. It usually took longer to take blood from alligators with their LAo intact (Figure 4.7). These animals were larger than alligators with their LAo cut and catching them, taping the mouth and taking blood took longer on average.

Larger alligators are stronger and so more difficult to “grab and stab”. Being aware of this, we tested for significant differences in the time it took to take blood between the two groups of alligators and also for significant effects of time on PO_2 , PCO_2 and pH values.

Busk *et al.* (2000b) found significant changes in arterial PCO_2 and HCO_3^- in alligators following feeding. They used indwelling catheters during their study to minimize stress and their alligators showed less pronounced pH changes following feeding than those observed by Coulson *et al.* (1950). The changes in plasma HCO_3^- were larger and remained elevated for a longer period in the study by Busk and colleagues. Their alligators ingested, on average, 7.5% of total body mass in food, whereas our alligators only ingested 4.31% and 2.79% of total body mass for alligators with their LAo intact and LAo cut, respectively. However, Busk and colleagues did not find a correlation between meal size and the magnitude of the alkaline tide and thus attribute differences in acid-base status between studies to blood sampling techniques. Thus, differences in the acid-base status following feeding between their study and ours could be due to the difference in blood sampling technique.

Studies have shown that average PCO_2 values increase after feeding (Weber and White, 1986; Busk *et al.*, 2000b). The small increase in PCO_2 following feeding is most likely due to a relative hypoventilation in which the ratio of $\dot{V}E/\dot{V}CO_2$ is reduced after feeding in order to compensate for the metabolic alkalosis after feeding. A study by Wang *et al.* (2003) showed that increased arterial PCO_2 values are directly related to the metabolic alkalosis experienced during digestion. In their study, gastric acid secretion

was inhibited by the oral administration of Omeprazole which inhibits gastric H^+,K^+ -ATPase, the proton-transporting enzyme in the parietal cells that secrete HCl into the stomach in snakes. This resulted in a reduced alkaline tide and thus eliminated the increase in arterial PCO_2 during digestion. They concluded that the increased PCO_2 after a meal is a method to maintain arterial pH.

Whole venous blood PO_2 values did not change significantly in both groups of alligators. A decrease in whole venous blood PO_2 results from an increased extraction rate as O_2 consumption rates of the tissues are increased. However, we did not observe a significant reduction in whole blood venous PO_2 values following feeding in both groups of alligators. There was also no significant difference in PO_2 values between the two groups of alligators, suggesting similar O_2 consumption rates by the tissues. Overgaard and Wang (2002) found that arterial PO_2 values remained high during digestion in *Python molurus*. They also predicted that venous PO_2 would be reduced during digestion due to the increased extraction rates of the tissues. Increased arterial PO_2 values after feeding have also been found in alligators (Busk *et al.*, 2000b) suggesting the absence of a shunt.

In the study by Busk *et al.* (2000b) and Weber and White (1986), alligators experienced a metabolic alkalosis as indicated by increased bicarbonate levels following feeding. However, our alligators did not show significant changes in HCO_3^- or Cl^- values after feeding. Changes in HCO_3^- and Cl^- levels are due to HCl secretion in the stomach. Average changes in HCO_3^- and Cl^- values were not significantly different from pre-feeding values and were not significantly different between the two groups of alligators

in our study. Because the changes in HCO_3^- and Cl^- were similar in both groups of alligators, HCl secretion during the time of blood sampling may also have been similar. We also have found no significant differences in digestion rates between the two groups of alligators (Chapter 2), suggesting similar gastric acid secretory rates.

Jones (1996) suggested that shunting RV blood to the body may aid in the secretion of HCl and/or in ameliorating the alkaline tide after feeding. By shunting RV blood to the digestive system, PCO_2 values could be increased due to the more acidic nature of RV blood. Also, an increase in serosal PCO_2 could enhance acid secretion by the gastric mucosa as was found in vitro by Kidder (1976) and Kidder and Montgomery (1974). For example, Kidder (1976) found that gastric acid secretory rates were limited by CO_2 availability in the dogfish gastric mucosa.

Alligators with their LAo intact have the ability to shunt RV blood to the body after feeding and thus lower average pH values were expected in that group. Average $\dot{V}\text{CO}_2$ values (Chapter 2) were significantly higher in alligators with their LAo cut, as were average RER values. Alligators with their LAo intact are able to shunt RV to the body via the LAo and this may be reflected in the lower $\dot{V}\text{CO}_2$ and higher average blood PCO_2 values. Alligators with their LAo cut are not able to shunt RV blood via the LAo and thus have higher $\dot{V}\text{CO}_2$ and lower average PCO_2 values. However, differences between the two groups of alligators were not significant and alligators with their LAo cut are able to adjust blood PCO_2 values by altering their relative ventilation.

So, why were we unable to measure any significant changes in acid-base status after feeding in our alligators? This could certainly be due to the small sample size of

our animals and due to our blood sampling technique i.e. grab and stab. Small sampling sizes will result in large variations of the data within an animal and between animals. Also, our blood sampling time greatly affected blood pH and gas measurements. The combined effects of a small a sample size and blood sampling technique could mask any real changes within each group and may mask and significant differences between the two groups of alligators. However, the trend observed was that alligators with their LAo cut tended to have higher average pH values throughout which lead us to the next experiment of changing ambient and water temperatures and thus intensifying changes in acid-base status.

The effect of temperature on acid-base status has been studied in many ectotherms, with the first work on alligators by Austin *et al.* (1927). The general trend observed is that with an increase in temperature, pH decreases and PCO_2 increases (Jackson and Kagen, 1976; Reeves, 1977; Davies, 1978; Davies *et al.*, 1982; Heisler, 1986; Weber and White, 1986). It is thought that ectothermic air-breathers adjust blood PCO_2 in response to temperature changes via adjustments in ventilation, particularly the air convection requirement (ACR; relative ventilation; $\dot{V}\text{E}/\dot{V}\text{O}_2$; $\dot{V}\text{E}/\dot{V}\text{CO}_2$). As temperature decreases, ACR increases to adjust the required PCO_2 , which ensures maintenance of protein charge (Reeves, 1972, 1977). This has been described as the imidazole alphastat (α -imidazole) hypothesis to explain the increase in pH and decrease in PCO_2 with decreasing body temperature in ectotherms. Thus, body pH is regulated in these animals to match the temperature dependent changes of the pK of the histidine imidazole groups. Several studies have shown that this occurs in alligators (Austin *et al.*,

1927; Davies, 1978; Davies *et al.*, 1982). For example, Davies *et al.*, (1982) showed that both $\dot{V}E/\dot{V}O_2$ and $\dot{V}E/\dot{V}CO_2$ decreased in alligators with an increase in body temperature. In their study, arterial PCO_2 increased and pH decreased with increased temperature and alligators maintained their protein charge with changing temperatures, i.e. maintained a constant α -imidazole charge.

In both groups of alligators, whole venous blood pH increased significantly as body temperature decreased and average PCO_2 values decreased. Although the response to changing temperature was similar in both groups of alligators, alligators with their LAo cut had significantly higher pH values and had lower PCO_2 values at 30° C and after one week at 20° C. We calculated the slopes of $\Delta pH/\Delta^\circ C$ for both groups of alligators and found that the slope was steeper for alligators with their LAo cut, implying a more pronounced response to changing temperature. The $\Delta pK/\Delta^\circ C$ for imidazole-like buffers is about -0.021 U/°C and it has been found that ectothermic animals on average show a $\Delta pH/\Delta^\circ C$ of about -0.011 U/°C, which is lower than $\Delta pK/\Delta^\circ C$ of free histidine-imidazole, thus α is not maintained (Heisler, 1986). In a study by Douse and Mitchell (1991), arterial pH values changed from an average of 7.48 at 30° C to an average of 7.62 at 20° C and back to 7.47 at 30° C. In our study, alligators with their LAo intact had average whole venous pH values of 7.27 at the initial 30° C, an average of 7.64 at 20° C and an average of 7.43 when at 30° C after the cold temperature exposure. Alligators with their LAo cut had average whole venous pH values of 7.50 at the initial 30° C, an average of 7.80 at 20° C and an average of 7.56 when at 30° C after the cold temperature

exposure. So, alligators with their LAo cut have on average more alkaline pH values. In the study by Douse and Mitchell (1991), $\Delta\text{pH}/\Delta^\circ\text{C}$ was about $-0.011 \text{ U}/^\circ\text{C}$ (from 30°C to 20°C) for alligators. In our study, alligators with their LAo intact had a $\Delta\text{pH}/\Delta^\circ\text{C}$ of $-0.0224 \text{ U}/^\circ\text{C}$, which is close to the value of $-0.021 \text{ U}/^\circ\text{C}$ for free histidine-imidazole. Alligators with their LAo cut had a $\Delta\text{pH}/\Delta^\circ\text{C}$ of $-0.0307 \text{ U}/^\circ\text{C}$, which is higher than $\Delta\text{pK}/\Delta^\circ\text{C}$ for free histidine-imidazole. From the equation $\alpha = 10^{(\text{pH}-\text{pK})}/(1 + 10^{(\text{pH}-\text{pK})})$ it appears that alligators with the LAo intact keep α constant but alligators with the LAo cut experienced larger pH disturbances and thus may not maintain a constant protein charge. Our slopes are steeper than those found by Douse and Mitchell (1991). Again, differences between studies could be due to differences in sampling technique.

A more acidic pH could be due to shunting RV blood to the body during the cooler temperature. Smith (1975a) suggested that alligators may employ the shunt during warming. He has also found that cutaneous blood flow is reduced during cooling in alligators (Smith, 1975b) and so our alligators may have employed the shunt during the cooler temperature in order to warm themselves. However, our whole venous blood PCO_2 values do not show any significant differences between the two groups of alligators after 3 weeks at 20°C . Also, water and room temperature were maintained at 20°C , so our alligators could not bask during that time and body temperatures were similar during the 20°C exposure.

Both groups showed similar responses to the change in body temperature, a metabolic alkalosis combined with a respiratory alkalosis (Figure 4.10). Davies (1978) showed that an increase in temperature caused ACR to decrease in alligators with both

$\dot{V}E/\dot{V}O_2$ and $\dot{V}E/\dot{V}CO_2$ falling. During lower temperatures $\dot{V}E/\dot{V}O_2$ and $\dot{V}E/\dot{V}CO_2$ increase. These adjustments in relative ventilation are thought to ensure the appropriate pH in order to maintain a constant α -imidazole charge.

Digestion is slowed or incomplete at temperatures lower than 25° C in alligators and caimans (Coulson and Coulson, 1986; Coulson *et al.*, 1990). For example, in *Caiman crocodilus*, when fed 5% of food of total body mass, complete gastric emptying took an average of 99 hours at 30°C and an average of 315 hours at 15° C. Prolonged digestive processes may be reflected in observed HCO_3^- levels. We found that both groups of alligators experienced a pronounced metabolic alkalosis at the lower temperature. HCO_3^- values increased initially as temperature was decreased and then decreased again in both groups of alligators (Figure 4.8). Fluctuations in HCO_3^- with a change in temperature have also been observed in the lizard *Tupinambis nigropunctatus* (Heisler and Neuman, 1976). They observed an initial increase in HCO_3^- after the temperature was lowered. Thereafter, HCO_3^- levels fluctuated during the whole measuring period. These changes in HCO_3^- may be due to an excretory mechanism or transcellular ion exchange processes using HCO_3^- .

The oxygen dissociation curve is affected by temperature, i.e. an increase in temperature causes the curve to be shifted to the right and the reverse is true for a decrease in temperature. Increasing the temperature denatures the bond between oxygen and hemoglobin, which increases the amount of oxygen and hemoglobin and decreases

the concentration of oxyhemoglobin (Schmidt-Nielsen, 1997) and the dissociation curve shifts to the right, causing a decrease in the affinity of hemoglobin for oxygen. Average PO_2 values decreased in both groups of alligators with a decrease in temperature (Figure 4.6). In animals with P→S shunts, arterial oxygen content is also a function of the shunt and will depend on the degree of shunting (Wood, 1984). A decrease in blood oxygen affinity at elevated temperatures will produce higher PO_2 levels and the reverse is true at lower temperatures. Even though the oxygen content of venous blood will be determined by the rates of oxygen delivery and oxygen demand by the tissues, a decrease in oxygen affinity at higher temperatures would result in higher venous PO_2 levels and vice versa at lower temperatures. We measured whole venous blood PO_2 values in our alligators and found no significant differences between the two groups of alligators at both temperatures. The changes in blood PO_2 were due to changes in temperature and do not indicate that alligators with their LAo intact employed the shunt during that time. Also, when temperatures were returned to 30° C, alligators with their LAo intact showed, on average, higher whole venous PO_2 values indicating that the shunt was not employed. It has to be noted that alligators get very upset during blood sampling, especially when repeated samples are taken, and alligators do not shunt when upset (personal observation). Our measured variables certainly reflect this and differences between groups may be masked by this fact.

However, average pH values were significantly higher in alligators with their LAo cut during the temperature experiments. Blood oxygen affinity in crocodilians is modulated by HCO_3^- and an increase in HCO_3^- could aid in O_2 unloading to the tissues

due to the unique effect of HCO_3^- on oxygen affinity in crocodilians (Weber and White, 1986). Alligator hemoglobin can bind two HCO_3^- molecules that act to reduce oxygen affinity (Bauer *et al.*, 1981; Weber and White, 1986; Jensen *et al.*, 1998). This could be of benefit during elevated temperatures when metabolic rates are elevated. At the lower temperature, while digesting food, elevated HCO_3^- levels may aid to unload O_2 to the digesting tissues, thus counteracting the effect of temperature on blood oxygen affinity.

The acid-base status of alligators was significantly affected in our studies, during the temperature experiments. Alligators with their LAo cut experienced significantly higher average pH values at 20° C. If this increase in pH is solely due to the effect of temperature on pH in alligators or due to a combination of temperature and digestion is not known in our study. However, the significant difference in average pH values disappeared when we stopped feeding alligators. Alligators with their LAo intact, however, continued to experience elevated average pH values. It appears that when alligators experience physiological challenges following feeding and during digestion, their acid-base status is significantly affected, which could have important implications for survival. During times of increased metabolic demands such as temperature changes or increased metabolic rates (running, fighting, mating) which cause changes in acid-base status, alligators with the LAo intact may be able to cope better by being able to regulate these acid-base disturbances more efficiently.

Figure 4.2. Changes in whole venous blood pH and PCO₂ (mm Hg) during long-term fasting in two groups of alligators. LAo intact: N=4; LAo cut: N=6. Values are presented as Means $\pm$ SEM. Significant differences between groups are indicated with an asterisk. Significant differences within each group from starting values (6 weeks) are indicated with a cross for alligators with their LAo intact and with a double cross for alligators with their LAo cut.

Figure 4.3. Changes in whole venous blood pH, PCO₂ (mm Hg) and PO₂ (mmHg) values following feeding in two groups of alligators. LAo intact: N=7; LAo cut: N=6. Values are presented as Means ± SEM. Significant differences between groups are indicated with an asterisk.

Figure 4.4. Changes in whole venous blood bicarbonate (HCO_3^- ; calculated, mmoles/liter) and Cl^- (mEq/liter) following feeding in two groups of alligators. LAO intact: N=6; LAO cut: N=5. Values are presented as Means $\pm$ SEM.

Figure 4.5. Davenport diagrams with calculated PCO_2 isoclines showing changes in whole venous blood acid-base status after feeding in two groups of alligators. The numbers by the data points indicate the time (hours) after feeding. Numbers next to the isoclines are the PCO_2 values (mmHg). LAo intact: $N=7$; LAo cut: $N=6$. Values are presented as Means $\pm$ SEM.

Temperature (°C) and weeks

Figure 4.6. Changes in whole venous blood pH, PCO₂ and PO₂ during 30° C and 20° C exposure in two groups of alligators. Numbers in parentheses are weeks of exposure at the particular temperature. BT – average body temperature of alligators during blood taking. LAO intact: N=5; LAO cut: N=4. Values are presented as Means $\pm$ SEM. Significant differences between groups are indicated with an asterisk. Purple and pink bars indicate if alligators were fed during that period.

Figure 4.7. Average times (sec) and the times at the two different temperatures to take blood from the cervical sinus during 30° C and 20° C exposure in two groups of alligators. Numbers in parentheses are weeks of exposure at the particular temperature. LAo intact: N=5; LAo cut: N=4. Values are presented as Means $\pm$ SEM. Significant differences between groups are indicated with an asterisk.

Figure 4.8. Changes in whole venous blood bicarbonate (HCO_3^- ; millimoles/liter; calculated) during 30° C and 20° C exposure in two groups of alligators. Numbers in parentheses are weeks of exposure at the particular temperature. LAo intact: N=5; LAo cut: N=4. Values are presented as Means $\pm$ SEM. Significant differences between groups are indicated with an asterisk.

Figure 4.9. Average pH and food intake (% per total body mass per group eaten one week prior to blood taking) during 30° C in two groups of alligators. LAo intact: N=5; LAo cut: N=4. Values are presented as Means $\pm$ SEM.

Figure 4.10. Davenport diagrams with calculated PCO_2 isoclines showing changes in whole venous blood acid-base status at different temperatures. The numbers by the data points indicate the respective temperatures. Numbers next to the isoclines are the PCO_2 values (mmHg). LAo intact: N=5; LAo cut: N=4. Values are presented as Means $\pm$ SEM.

CHAPTER 5

Summary and conclusions

The central hypothesis of this thesis was that shunting right ventricular (RV) blood to the digestive system aids in digestion and absorption of nutrients and/or in reducing the effects of the alkaline tide following feeding (Figure 5.1). The synthesis of HCl by the parietal cells requires CO_2 and in the process HCO_3^- is produced. The PCO_2 in gastric juice exceeds the PCO_2 of blood and CO_2 used to synthesize HCl may originate from the blood perfusing the stomach. Stevens *et al.* (1987) provided two potential explanations for this: (1) that CO_2 diffuses from the blood into the stomach lumen against a concentration gradient or (2) that CO_2 is produced within the gastric lumen from HCO_3^- ($\text{H}^+ + \text{HCO}_3^- \leftrightarrow \text{H}_2\text{O} + \text{CO}_2$). Kidder (1976) and Kidder and Montgomery (1974) found that acid secretory rates by the gastric mucosa in vitro were limited by CO_2 availability in dogfish and frogs. Thus, shunting blood high in CO_2 to the gut during digestion may aid in the synthesis of HCl and/or in ameliorating the alkaline tide, i.e. the increase in HCO_3^- following feeding. We found that during shunting periods, microspheres injected into the LAo were primarily found in organs associated with digestion such as stomach, small intestine, pancreas and gallbladder (Figure 1.7). We also found that the kidneys primarily received LAo blood (i.e. RV blood) during shunting periods. Digestion in alligators takes a long time and thus gastric pH levels remain low for long periods of time (Figure 1.9). Alligators are intermittent breathers and thus reliance on ventilatory adjustments to modify long-term blood acid-base variables (pH, PCO_2) may not always be feasible. However, we found that alligators may shunt for long periods of time and so

shunting RV blood to the gut during digestion could be a strategy to adjust acid-base variables.

My prediction was that digestion and absorption would be impaired and/or prolonged in alligators that are unable to shunt RV blood to the digestive system during feeding. I also predicted that acid-base changes after feeding would be more pronounced in alligators without the ability to shunt RV blood to the digestive system. The ability to shunt RV blood to the body during fasting did not benefit alligators. All variables measured during that time were similar in the two groups of alligators. Differences in acid-base status were observed at the beginning of the fasting period (Chapter 4). The initial differences in whole venous blood pH and PCO_2 values could have been due to differences in digestive state between the two groups of alligators. Unfortunately, we were not able to separate the two groups of alligators during that study and thus could not determine the amount of food eaten per group. If the food intake was larger in alligators with their LAo cut, this could be reflected in differences in acid-base status, i.e. more alkaline pH values at the beginning of the fasting period.

Growth rates of our alligators were not significantly affected by ligating the LAo. Both groups increased in mass and length at similar rates and had similar conversion rates (Chapter 2). Oxygen consumption rates were not significantly different between the two groups of alligators. This implies that the metabolic rates of the tissues would be similar during digestion. We also found that whole venous blood PO_2 values were similar in both groups of alligators (Chapter 4). Average $\dot{\text{VCO}}_2$ and RER values were higher in alligators with their LAo cut because all blood must pass through the lungs. However, digestion rates were not significantly different between the two groups of alligators,

indicating that HCl secretion was adequate in both groups. Thus, it seems unlikely that shunting RV blood to the digestive system has significant effects on HCl secretion in alligators. During non-shunting times the digestive system receives oxygenated blood via the anastomosis (Figure 5.1). During shunting times, the digestive system will receive oxygenated blood via the anastomosis during early systole and RV blood during late systole. Thus, the digestive organs receive a mixture of LV and RV blood during shunting. The oxygen and carbon dioxide contents of the blood perfusing the digestive system therefore depend on factors such as cardiac output and relative blood flow in the blood vessels (RAo, LAo, anastomosis, gastric artery, etc.).

More insight was gained when the acid-base status of the two groups of alligators was evaluated (Chapter 4). The trend observed was that alligators with their LAo cut had higher whole venous blood pH and lower PCO₂ values during feeding. Even though these differences were not statistically significant, this trend was observed throughout the studies. This was also observed at the beginning of the fasting period (Chapter 3) and when we exposed alligators to a low temperature of 20° C (Chapter 4).

Alligators can affect their blood pH and gas levels via adjustments in respiration and via the P→S shunt. During feeding alligators can experience a large alkaline tide (Coulson *et al.*, 1950). Our results showed no significant changes in pH following feeding. It has been suggested that the effects of the alkaline tide may be compensated by increased PCO₂ levels following feeding due to retention of CO₂ either by reduced pulmonary exchange or by shunting high CO₂ blood to the body. Alligators hypoventilate after feeding and this is reflected not only in measured blood PCO₂ values but also in their air convection requirements. Alligators could use the P→S shunt to

compensate for the metabolic alkalosis by shunting blood to the body that is relatively high in CO_2 and thus reducing the effects of the alkaline tide. Changes in ventilation, as well as shunting RV blood to the digestive system could be employed simultaneously, resulting in lower than expected RER values due to low $\dot{V}\text{CO}_2$ values and in higher blood PCO_2 values.

Alligators with their LAo cut had higher whole venous blood pH values and lower PCO_2 values during lower body temperatures. Not being able to employ the P→S shunt may result in higher average blood pH values, lower blood PCO_2 values and higher $\dot{V}\text{CO}_2$ values because alligators with their LAo cut can not shunt RV blood to the digestive system via the LAo and all the blood has to go through the lungs.

We ligated the LAo just outside the heart in one group of alligators, preventing shunting of RV blood to the digestive system via the LAo. However, these animals may still be able to shunt RV blood to the body via the foramen of Panizza. Flow through the foramen of Panizza may occur from the LAo into the RAo (Axelsson *et al.*, 1995; Axelsson and Franklin, 1997). Axelsson *et al.* (1995) showed in their study that the left aortic valves do not completely cover the foramen of Panizza, so the possibility of flow from the LAo to the RAo remains. Karila *et al.* (1985) showed that the foramen is surrounded by smooth muscle and highly innervated, suggesting that the diameter may be of variable size depending on physiological state. This was also suggested by Grigg and Johansen (1987). Whether our alligators utilized the foramen of Panizza to shunt RV blood to the body during our study is unknown. RV blood shunted to the body via the foramen would be sent to the body via the RAo, which carries LV blood. The digestive system in this case would receive this mixed blood via the anastomosis from the RAo.

Due to the small flow into the LAo through the foramen (its size is only 30-40% of right aortic diameter; Axelsson *et al.*, 1996), the impact on the blood gas composition (PCO_2 and PO_2) of blood entering the digestive system would be small. Jones and Shelton (1993) showed that when completely closing off the pulmonary artery and thus preventing any flow to the lungs, a P→S shunt was induced as indicated by an increased flow. Flow within the RAo at that time was greatly reduced. This was also demonstrated in that same study by injecting acetylcholine in the RAo of an unanaesthetized alligator. Left aortic pressure increased above that of RAo pressure but flow within the RAo did not increase. These types of flow patterns and pressure changes could only be induced by complete obstruction of the pulmonary circulation and that during a natural shunt (also recorded by Jones and Shelton, 1993) flow to the lungs is not compromised and occurs during early systole. Thus, it is unlikely that flow through the foramen of Panizza from LAo to RAo was occurring in our alligators.

There has been great debate on the functional significance of shunting in crocodilians (for review see Hicks, 1998; Hicks and Wang, 1996; Axelsson and Franklin, 1997; Hicks, 2002; Wang *et al.*, 2001b). For example, it has been suggested that an important role of the P→S shunt in crocodilians may be to sequester CO_2 away from the lungs during diving (Grigg and Johansen, 1987; White, 1978). This would facilitate O_2 uptake in the lungs during apneic periods and promote O_2 delivery in the tissues (a right Bohr shift). However, several studies have shown that crocodilians may not always shunt during voluntary dives (Grigg and Johansen, 1987; Smith *et al.*, 1974). White (1956) showed that alligators developed the shunt during forced diving. However, we found that alligators may shunt almost continuously when quiescent and relaxed, a manner that may

be observed during digestion. Also, studies have shown that alligators and caimans do not necessarily exhibit a bradycardia during diving (Gaunt and Gans, 1969; Smith *et al.*, 1974; Weinheimer *et al.*, 1982). We have found no correlation of ventilatory state and shunting in our laboratory (unpublished observation). In alligators, once the shunt is fully operative, the shunt continues regardless of ventilatory state (breathing versus apnea). This has also been observed in turtles (Wang *et al.*, 1997).

Farmer and Carrier (2000) exercised alligators and found low RER values following recovery from exercise. They suggested that the P→S shunt may be used to ensure O₂ unloading in the tissues due to the Bohr effect (right shift) and that the shunt may facilitate CO₂ retention during recovery, replenishing bicarbonate buffer stores and restoring blood pH while still enabling O₂ uptake in the lungs after exercise. This would be reflected in low RER values which were 0.3 in their study. Several studies have shown that the R→L shunt is reduced during activity, higher body temperatures or digestion in some reptiles when demands for oxygen are high in the tissues (for review see Wang *et al.*, 2001b).

Hicks (2002) suggested that the retention of the shunt may be a relic. He argued that due to the low metabolic rates in ectotherms, the shunt does not pose a problem and thus would not affect survival in these animals. To date, there still is no definite answer to explain why crocodilians have retained the ability to shunt RV blood to the digestive system via the LAo.

It is obvious that ligating the LAo did not affect the survival of our alligators. The response to different physiological states (feeding, change in temperature) was similar in both groups of alligators. When challenged with low temperatures, average pH

values were significantly higher in alligators with their LAo cut. Our alligators continued to eat at the low temperature and the elevated pH values were most likely a result of the prolonged digestion during the lower temperature, as well as the effect of decreased temperature on pH in these animals (Chapter 4). During times of increased physiological demands such as exercise, mating, defending the territory and temperature changes, acid-base regulation may become more difficult in alligators without the ability to utilize the P→S shunt. In the python, $\dot{V}O_2$ and $\dot{V}CO_2$ were significantly higher during post-feeding exercise than following feeding or exercise alone (Secor *et al.*, 2000) and similar results have been observed in *Varanus* (Hicks *et al.*, 2000). Digestion and exercise have different effects on the acid-base status and these animals may adjust their ventilation as well as blood flow patterns to deal with these changes. It is important to study the effects of ligating the LAo of free ranging alligators and crocodilians as they experience physiological challenges on a daily basis. For example, wild alligators are subjected to a wide range of temperatures, ranging from 5° C to 37° C (Coulson and Hernandez, 1983). Alligators feed at the higher temperatures and some studies have shown that they stop feeding at temperatures below 25° C, resulting in fasting periods lasting for months. During times of feeding, exposure to different temperatures and fasting, an alligator undergoes a series of physiological adjustments such as changes in acid-base status and changes in gastrointestinal morphology during fasting (atrophy of the gut).

To date, we still do not have a clear picture on when alligators shunt in nature. In the laboratory, these animals stop shunting as soon as they are disturbed or stressed. Consequently, it is of vital importance to attain blood flow measurements when the animals are behaving normally in a natural setting. Using telemetric devices such as our

intragastric pH probe in combination with blood flow devices will provide more insight into the functional significance of shunting (if any) during different physiological states in alligators.

Figure 5.1. Drawing showing blood flow during non-shunting and shunting periods in the alligator heart (see Chapter for details) and simplified drawing of a parietal cell. The blue arrows indicate reactions favored due to shunting RV blood to the body during digestion.

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