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A metabolic hypothesis for the evolution of temperature effects on the arterial PCO_2 and pH of vertebrate ectotherms

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Summary Statement: We develop an additional hypothesis to protein structure-function relations for the increase in arterial PCO_2 and decline in blood pH with increases in body temperature of ectotherms.

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Abstract

Body temperature increases in ectothermic vertebrates characteristically lead to both increases in arterial PCO_2 ($PaCO_2$) and declines in resting arterial pH (pHa) of about 0.017 pH units/°C increase in temperature. This 'alphastat' pH pattern has previously been interpreted as being evolutionarily-driven by the maintenance of a constant protonation state on the imidazole moiety of histidine protein residues, hence stabilizing protein structure-function. Analysis of the existing data for interclass responses of ectothermic vertebrates show different degrees of $PaCO_2$ increases and pH declines with temperature between the classes with reptiles > amphibians > fish. The $PaCO_2$ at the temperature where maximal aerobic metabolism (VO_{2max}) is achieved is significantly and positively correlated with temperature for all vertebrate classes. For ectotherms, the $PaCO_2$ where VO_{2max} is greatest is also correlated with VO_{2max} indicating there is an increased driving force for CO_2 efflux that is lowest in fish, intermediate in amphibians and highest in reptiles. The pattern of increased $PaCO_2$ and the resultant reduction of pHa to increased body temperature would serve to increase CO_2 efflux, O_2 delivery, blood buffering capacity and maintain ventilatory scope. This represents a new hypothesis for the selective advantage of arterial pH regulation from a systems physiology perspective in addition to the advantages of maintenance of protein structure-function.

Introduction

Body temperature influences blood acid-base balance in a very predictable pattern in ectothermic vertebrates, with a decrease of about 0.017 pH units/°C increase in temperature (Howell et al., 1970; Reeves, 1972). The regulation of ventilation with temperature has been proposed as a mechanism to regulate arterial PCO_2 ($PaCO_2$) and thus arterial pH (pHa) with temperature changes in ectotherms (Glass et al. 1985). In most ectotherms studied, increased ventilation does not match the temperature-induced increase in metabolism, and this relative hypoventilation leads to an increase in $PaCO_2$ that decreases pHa from the generation of carbonic acid. Interestingly, this pattern parallels the effect of temperature variation on the pH of water. The rate of change in pH for both water and pHa is about -0.017 pH units/°C, and because the arterial blood of ectotherms is about 0.6 pH units greater than that of water at any temperature, the phenomenon was frequently referred to as maintaining 'relative alkalinity' (Rahn, 1967), and the regulatory process to achieve this as alphastat pH regulation (Reeves, 1972). The prevailing hypothesis for the advantage of alphastat pH regulation is maintenance of a constant ratio of OH^- to H^+ despite variation in pH. This alphastat pH pattern maintains a constant fractional protonation state on the imidazole moieties of histidines in proteins (Reeves, 1972,1977). This has been argued to better maintain protein structure and function and preserve cellular function with varying body temperatures.

Reeves' hypothesis for alphastat regulation of blood pH suggests that ventilation, and thus $PaCO_2$, is regulated to maintain a constant fractional dissociation of histidine imidazole residues on proteins. This hypothesis implies that the change in pH with

temperature is regulated to equal the change in the pK with temperature of the imidazole buffer system, which is about -0.018 to -0.024 U/°C (Edsall and Wyman, 1958). Although there is some support for the alphastat hypothesis for regulation of blood pH in ectotherms, there are several studies showing that the change in blood pH with temperature is significantly lower than the change in pK with temperature required for alphastat pH regulation (see Glass et al. 1985). Thus, although alphastat regulation is an attractive hypothesis for explaining the pattern of blood pH regulation in ectotherms, Cameron (1989) pointed out that as a realistic predictor of protein behavior, alphastat needs to be revised to accommodate both advances in protein chemistry and the evident heterogeneity of physiological findings. The pattern of increased $PaCO_2$ and decreased pHa with increasing temperature has also been interpreted as a means of depressing metabolism via ventilation during bouts of torpor or hibernation in both endotherms and ectotherms (Malan, 2014).

Given the heterogeneity of the physiological data and in an attempt to provide an integrative metric of organismal function, we present an argument for the consideration of an organ system level advantage related to O_2 and CO_2 fluxes during periods of increased aerobic demands associated with both increased temperature and activity for an increase in the regulated $PaCO_2$ and consequential decrease in pHa with increases in temperature. Standard and maximal rates of aerobic metabolism of all ectotherms are temperature sensitive, with a range of Q_{10}'s of about 1.5-3 (Hedrick et al. 2015). Maximal rates of aerobic metabolism during activity at an organ system level reflect the maximal rates of oxygen delivery to working muscle and the maximal rates of CO_2 removal from working muscle to the environment. The cardiovascular system is the principal limitation

to maximal oxygen delivery in vertebrates (Hillman et al., 2013), whereas the respiratory system appears to be the principal limitation to CO_2 efflux in ectotherms (Hillman et al., 2013; Hedrick et al., 2015). Consequently, co-adaptations that enhance the capacity for both enhanced O_2 delivery and CO_2 efflux will enhance aerobic metabolic capacity.

There is a shift in $PaCO_2$ and pH regulation in the evolutionary transition from fish to amphibians and reptiles associated with the differences in O_2 and CO_2 capacitances of water and air (Dejours, 1975). Fish primarily regulate pH across their gills via ion exchangers (Na^+/H^+, Cl^-/HCO_3^-) but CO_2 is exchanged by diffusion (Heisler, 1986). Amphibians and reptiles primarily achieve pH regulation via ventilatory regulation of $PaCO_2$. From an organismal metabolic perspective how might an 'alphastat pH pattern' of reduced pH and increased $PaCO_2$ increase O_2 delivery and CO_2 removal with increases in temperature? We suggest that the regulated hypoventilation associated increased temperature would 1) preserve ventilatory capacity; 2) the resultant increase in $PaCO_2$ would increase the driving force for CO_2 efflux; 3) the increase in $PaCO_2$ would increase HCO_3^- and buffering capacity of the blood; and 4) the decrease in pHa would increase the delivery of O_2 (Bohr Effect) and the efflux of CO_2 (Haldane Effect) at both rest and during activity with increased body temperature.

If increasing the regulated $PaCO_2$ with increased body temperature is selectively advantageous for enhancing organ system gas exchange, there are a variety of predictions that might follow: 1) increased temperature should increase $PaCO_2$ and decrease pHa within the different classes of ectothermic vertebrates, and 2) interclass variation of the $PaCO_2$ responses to temperature should correlate with interclass variation of the aerobic metabolic capacity. If these predictions hold, it suggests that there may be an alternative

or additional evolutionary explanation to protein structure-function driving the evolution of this alphastat pH pattern of changes in $PaCO_2$ and pH with temperature.

Materials and Methods

Venous PCO_2 ($PvCO_2$) directly reflects the actual driving force for CO_2 diffusional efflux across the respiratory surface assuming that alveolar PCO_2 remains the same. The difference between $PvCO_2$ and $PaCO_2$ is small at rest and in many cases almost indistinguishable, but resting $PaCO_2$ represents a minimal estimate of the potential driving force across the respiratory surface. There are more data available for resting $PaCO_2$ than $PvCO_2$, thus we have used resting $PaCO_2$ values throughout in our analysis. Although using resting $PaCO_2$ may underestimate the actual driving force for CO_2 efflux, especially during activity, increases in $PaCO_2$ clearly reflect physiologically regulated increases in the net driving force for PCO_2 efflux.

To evaluate the consistency of both blood pH (pHa) and $PaCO_2$ to temperature for each group of ectotherms we have used the summary data of Ultsch and Jackson (1996), which primarily selected data based on cannulated sampling rather than heart punctures for resting animals. Data for $PaCO_2$ of resting mammals and birds were taken from Lahiri (1975), Tenney and Boggs (1986), Gleeson and Brackenbury (1984), Cushing and McClean (2010), Murrish (1983), Ponganis et al. (2007), Peters et al. (2005) and Scott and Milsom (2007).

Metabolic data (resting and maximal) for each class were taken from the summaries within Hedrick et al. (2015). Aerobic generation of CO_2 is the result of aerobic metabolism and its efflux can be quantified as the product of conductance and the driving force for CO_2 (i.e. $G_{CO2} \times \Delta PCO_2$) Our hypothesis is that the increase in $PaCO_2$

with temperature reflects an increase in the physiologically regulated driving force for CO_2 efflux. Consequently, to test that the Q_{10} for the rate of resting CO_2 efflux should parallel the Q_{10} for the PCO_2 driving force, we calculated the ratio of resting $PaCO_2$ at different temperatures. We used the resting $PaCO_2$ regressions, summarized in Figures 1 and 2, to determine the ratio of $PaCO_2$ differences between two temperatures, analogous to the calculation of Q_{10} for reaction rates (i.e. (Rate 2/Rate 1)$^{10/(T2-T1)}$) or ($PaCO_2$ @ T2 /$PaCO_2$ @ T1) $^{10/(T2-T1)}$ (see Jackson, 1978).

Least squares regression was used to determine slopes and significance using Prism v. 5 (Graphpad software, Inc. La Jolla, CA (USA).

Results and Discussion

There were significant increases in resting $PaCO_2$ with increased temperature for fish ($F_{1,58}$ = 7.1; P= 0.0098; $PaCO_2$ (kPa) = 0.0067 (±0.002) °C + 0.246, r^2 = 0.11), amphibians ($F_{1,38}$ = 51.1; P< 0.0001; $PaCO_2$ (kPa) = 0.0538 (±0.008) °C + 0.0305, r^2 = 0.57), and reptiles ($F_{1,68}$ = 29.8; P<0.0001; $PaCO_2$ (kPa) = 0.0691 (±0.013) °C + 1.18, r^2 = 0.30) (Fig. 1A). The slope of this relationship for fish, although significant, was about 10-fold lower than the slope for amphibians or reptiles. This would be expected given the low $PaCO_2$ in fish due to the high CO_2 capacitance in water.

There was a significant effect of temperature (p<0.0001) on resting blood pH for fish ($F_{1,90}$ = 39.6; pH = 8.04 – 0.010 (±0.002) °C, r^2 = 0.31), amphibians ($F_{1,44}$ = 70.0; pH = 8.09 – 0.013 (±0.002) °C, r^2 = 0.61), and reptiles ($F_{1,78}$ = 258; pH = 7.96 – 0.014 (±0.001) °C, r^2 = 0.77) (Fig. 1B). Taken together, these results are consistent with a resting CO_2-mediated decrease in blood pH with increasing body temperature.

At any particular temperature, $PaCO_2$ for reptiles was approximately double that of amphibians, and amphibians were 3-4 times that of fish (Fig. 1A). The elevated $PaCO_2$ of reptiles would therefore account for the lower pH for this group at any body temperature (Fig. 1B).

The temperature at which VO_{2max} occurs is lowest in fish (20 °C), intermediate in amphibians (25 °C) and reptiles (35 °C), and highest in mammals and birds (see Fig. 4 in Hedrick et al. 2015). The temperature at which resting $PaCO_2$ corresponds with VO_{2max} for five vertebrate classes (Hedrick et al. 2015) is presented in Fig. 2A. There is a significant, linear relationship ($p<0.0077$; $r^2 = 0.93$) between $PaCO_2$ and the temperature at which VO_{2max} occurs indicative of an increased driving force for CO_2 efflux with increased temperature at VO_{2max} for these vertebrate groups.

The relationship between VO_{2max} and $PaCO_2$ where VO_{2max} occurs for all vertebrate groups is presented in Fig. 2B. Resting $PaCO_2$ increases with the greatest VO_{2max} for the ectothermic classes, but is independent of VO_{2max} in the endothermic classes. A plateau of approximately 5 kPa $PaCO_2$ seems to occur for vertebrates in general; reptiles at 35°C are near this apparent plateau.

Enhancing Ventilatory Scope

Our analysis of the resting $PaCO_2$ patterns with temperature in fish, amphibians and reptiles (Fig. 1) reveals that at a given temperature, resting $PaCO_2$ is greatest in reptiles, intermediate in amphibians and lowest in fish. There were significant increases in resting $PaCO_2$ with temperature in all three groups. The alveolar ventilation (V_A) equation predicts alveolar PCO_2, and thus $PaCO_2$, to be inversely related to the 'air convection requirement' (ACR) ratio in air-breathing ectotherms (i.e. V_I/VO_2 or V_E/VO_2)

and the increased $PaCO_2$ (and decreased pH) with temperature can be explained by an unequal response of minute ventilation (V_I or V_E) relative to metabolism. This approach would also apply to fish, substituting water for air. The hypoventilation (decreasing V_A) will increase the ventilatory scope available during activity. Assuming consistent interclass Q_{10} effects on metabolism the magnitude of the hypoventilation can be estimated as $V_A = 1/P_aCO_2$. The mean decrease in V_A for the temperature intervals from 10 °C to 20 °C and 20 °C to 30 °C for fish is 16%, for amphibians 34%, and 24% for reptiles. This estimate reflects the potential increase in ventilatory scope available to enhance gas exchange with activity than if these groups maintained a constant ACR and pHa. Although the alphastat hypothesis implies that the reduced ACR with increased temperature is necessary to maintain a constant fractional dissociation of imidazole residues, we suggest that the reduced ACR with temperature may also be important for preserving ventilatory capacity with increased metabolism associated with both temperature and activity.

There are additional arguments that support this hypothesis. First, the pattern of pH regulation we observed for fish, amphibians and reptiles in this study do not fit the traditional alphastat hypothesis proposed by Reeves (1972). The slopes for the change in pH with temperature for the air-breathing ectotherms, amphibians (-0.013 U/°C) and reptiles (-0.014 U/°C), were about 25-30% lower than the approximate -0.017 U/°C required for alphastat regulation, and similar to the values found previously for a number of reptile species (Glass et al. 1985). Second, previous work in reptiles has shown that V_I or V_E increases about 3-4 fold with a temperature increase from 10 °C to 30 °C whereas VO_2 increases 6-7 fold over the same temperature range (Funk and Milsom, 1987; Glass

et al. 1985). This is the basis for the reduced ACR, but if minute ventilation were matched to metabolism, thus maintaining a constant $PaCO_2$ and pH (i.e. pH stat regulation), the resulting increase in minute ventilation would leave less scope for further increases with increased temperature or during bouts of activity as described above. We showed previously (Hillman et al. 2013) that at maximal exercise, CO_2 extraction at the respiratory surface increases significantly in all vertebrates, and the ratio of V_I to blood flow at the respiratory surface increases about 3 fold to support increase of CO_2 extraction at VO_{2max}. This requires a ventilatory capacity from rest to activity to support the increased CO_2 extraction to maintain maximal CO_2 efflux. Even with this level of ventilatory increase, $PaCO_2$ increases at VO_{2max} in fish and amphibians indicating that ventilation does not keep pace with the needs for CO_2 efflux (Hillman et al. 2013).

Enhancing Bohr and Haldane Effects

The relative hypoventilation with increased $PaCO_2$ and reduced pHa pattern also takes advantage of Haldane and Bohr effects for increasing CO_2 and O_2 transport, respectively, with increased temperature. The delivery of O_2 from hemoglobin (Hb) is influenced by the decline in arterial pH since $O_2 + Hb \leftrightarrow HbO_2 + H^+$, hence by mass action an increase in the $[H^+]$ at the tissue level (from elevated PCO_2 and lactic acid) favors unloading of the Hb (Bohr Effect) and enhanced O_2 delivery at the muscle. The increase in $[H^+]$ also enhances the uptake of CO_2 at the tissue as a consequence of formation of carbamino CO_2 on the Hb molecule (Haldane Effect). The increase in $[H^+]$ also favors the release of CO_2 at the respiratory surface by mass action from the following reaction: $H^+ + HCO_3^- \leftrightarrow H_2O + CO_2$. The advantages of the Haldane and Bohr effects for gas transport would not be fully realized without the regulated increase of

$PaCO_2$ and reduced pHa in ectotherms. Although the increase in $PaCO_2$ and CO_2 efflux is due, in part, to adjustments in the ACR, the impact on O_2 transport are primarily caused by the right shift of the O_2 dissociation curve with increased temperature and reduced pH (Bohr effect), and its interaction with intracardiac shunts that increase PaO_2 and systemic O_2 transport. Taken together, we suggest that the regulated hypoventilation relative to metabolism provides several identifiable benefits to systems gas transport independent of any effects on alphastat pH regulation.

Enhancing the CO_2 Efflux Driving Force

As indicated above, CO_2 efflux is the product of G_{CO2} and ΔPCO_2. In order to increase CO_2 efflux with increased metabolic demands, either or both of these variables can be increased. For resting animals, the temperature-mediated ratios for the relationship of $PaCO_2$ with temperature are 1.2 for fish, 1.5-1.9 for amphibians and 1.2-1.4 for reptiles, all generally lower than the Q_{10}'s of 2-3 for standard and maximal metabolism (see summary in Hedrick et al. 2015). This indicates that changing the driving force for CO_2 efflux by raising $PaCO_2$ does not explain an intraclass limitation on VCO_2 with changes in temperature and, instead, suggests the potential for co-adaptations in respiratory conductance and/or ventilatory capacity. Based on the resting $PaCO_2$ values in Fig. 1A, the driving force for CO_2 efflux is increased 42% for fish, 128% for amphibians and 73% for reptiles with body temperature increasing from 10°C to 30°C. This indicates that the $PaCO_2$ response to temperature in each class would enhance the driving force for CO_2 efflux during maximal activity by increasing the regulated resting $PaCO_2$, but not sufficient to account for the Q_{10} during maximal activity.

An interesting intraclass test of the driving force hypothesis can be found in fish, a truly bimodal group (water versus air) in terms of gas exchange. The obligate air breathing four species of fishes in the summary of Ultsch and Jackson (1996) have a $PaCO_2$ of about 3.3 kPa compared to 0.42 kPa for water breathing fish at equivalent temperatures. We interpret this as the necessity to increase the driving force for CO_2 efflux when the gas bladder conductance is probably lower than the gill conductance and the decrease in CO_2 capacitance of air compared to water.

From a maximal aerobic metabolic perspective, what might be the effect of interclass variation in the magnitude of $PaCO_2$ response to increased temperature on the capacity to enhance O_2 delivery and CO_2 efflux during activity? Based on the data from Fig. 2A, the ratio of interclass $PaCO_2$, at their respective temperatures for VO_{2max}, between fish (20 °C) and amphibians (25 °C) is 17.1 and between amphibians and reptiles the interclass ratio is 2.5. The large phylogenetic ratio for $PaCO_2$ between fish and amphibians is consistent with the Q_{10} of 13.4 for VO_{2max} between fish and amphibians at 20 °C and 25 °C, respectively, and a Q_{10} of 1.2 for VO_{2max} between amphibians and reptiles at 25 °C and 35 °C, respectively (Hedrick et al. 2015). The correspondence of Q_{10} values between VCO_{2max} and the ratios for interclass $PaCO_2$ is consistent with an increase in $PaCO_2$ playing a significant role in explaining interclass variation in VCO_2max, unlike the resting condition where increased conductance (G_{CO2}) appears to provide the increase in resting VCO_2. As noted above, fishes and amphibians, increase $PaCO_2$ at VO_{2max} (see Hillman et al. 2013) which would enhance CO_2 efflux by increasing the driving force for PCO_2 to a greater extent than our estimates here using resting $PaCO_2$. For reptiles, $PaCO_2$ at VO_{2max} does not appear to increase over resting

values (Hillman et al. 2013), thus our estimates of CO_2 efflux based on resting $PaCO_2$ values for this group are probably more accurate.

These data may also indicate that increasing the $PaCO_2$ driving force to increase CO_2 efflux in vertebrates has limits. For example, increases of $PaCO_2$ greater than 5 kPa, which appears to be near the upper limit for reptiles and endotherms, may cause significant changes in pH that potentially compromise protein function, suggesting that endotherms use alternative adaptations such as increased respiratory conductance and ventilatory capacity to achieve the greater fluxes of O_2 and CO_2.

Enhancing the Blood Buffering Capacity

The increase in $PaCO_2$ also leads to increased concentrations of HCO_3^- (Ultsch and Jackson, 1996). An increase in $[HCO_3^-]$ would increase the buffering capacity of the blood. Lactic acid begins to accumulate in the blood when aerobic power outputs during activity are 50-70% of maximal (Davis et al., 1996, Seeherman et al., 1983, Gleeson and Brackenbury, 1984, Taigen and Beuchat, 1984, Goolish, 1991). Consequently, an added selective advantage of the increase in $PaCO_2$ and $[HCO_3^-]$ with increased VO_{2max} is less disruption of pHa during high metabolic power outputs. Malan (2014) has also suggested increased buffering as a benefit of the hypercapnic acidosis associated with hibernation and torpor.

Regulatory Mechanisms

The hypothesis presented here requires a linkage between body temperature and the regulation of ventilation. The regulation of increased $PaCO_2$ and reduced ACR with increased temperature implies a receptor linked to ventilation operates to maintain ventilation within narrow limits as temperature changes. It is well known that $PaCO_2$ is tightly regulated by the complex interactions of central and peripheral chemoreceptors in vertebrates (Milsom, 2002). A ventilatory-mediated mechanism that controls ventilation and, therefore, arterial $PaCO_2$ and pHa with changes in temperature provides a convenient negative feedback mechanism. Recent work with bullfrogs (*Lithobates catesbeianus*) and monitor lizards (*Varanus exanthematicus*) has shown the presence of CO_2/pH chemosensitive neurons of the locus coeruleus (LC), a putative ventilatory control region (Santin et al. 2013; Zena et al. 2016). *L. catesbeianus* has been characterized as a typical alphastat regulator (Reeves, 1972; Santin et al. 2013), whereas *V. exanthematicus* is a pH-stat regulator with little change in pHa over a broad range of temperatures (Zena et al. 2016). In *L. catesbeianus*, cooling increased, and warming decreased, the firing rate of LC chemosensitive neurons (Santin et al. 2013). Moreover, cooling reduced CO_2/pH chemosensitivity in a temperature-dependent fashion, thus the magnitude of the chemosensitive response was temperature-dependent (Santin et al. 2013). By contrast, chemosensitive LC neurons in *V. exanthematicus* increase firing rates with increasing temperature and have a large Q_{10} effect compared with bullfrog chemosensitive LC neurons (Zena et al. 2016). *V. exanthematicus* also have populations of LC neurons that are excited or inhibited by CO_2 and the proportion of CO_2-inhibited

neurons increases with cooling (Zena et al. 2016). The findings that populations of CO_2/pH chemosensitive neurons in the LC of bullfrogs and lizards that are modulated by temperature provides a parsimonious explanation for ventilatory regulation of $PaCO_2$ and pHa with changes in body temperature.

Conclusions

We suggest that the pattern of arterial pH and $PaCO_2$ initially described by Howell et al. (1970) and later interpreted from a solely biochemical structure/function perspective (Reeves 1972, 1977; White and Somero, 1982) may additionally, or primarily, have its evolutionary basis in the enhancement of systems level gas transport. Increased temperature increases aerobic demands for O_2 influx and CO_2 efflux both at rest and during activity. The alphastat pattern of hypoventilation relative to aerobic metabolic demand, leading to increases in $PaCO_2$ and $[HCO_3^-]$ and decline in pHa, preserves ventilatory capacity, increases blood buffering capacity and enhances both CO_2 and O_2 fluxes that would be associated with increases in body temperature and activity. We suggest this hypothesis deserves consideration along with potential (as yet undocumented) imidazole-mediated protein structure-function considerations.

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Competing Interests

The authors declare no competing financial interests.

Author contributions

Each author contributed equally to the development and writing of the manuscript.

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Figures

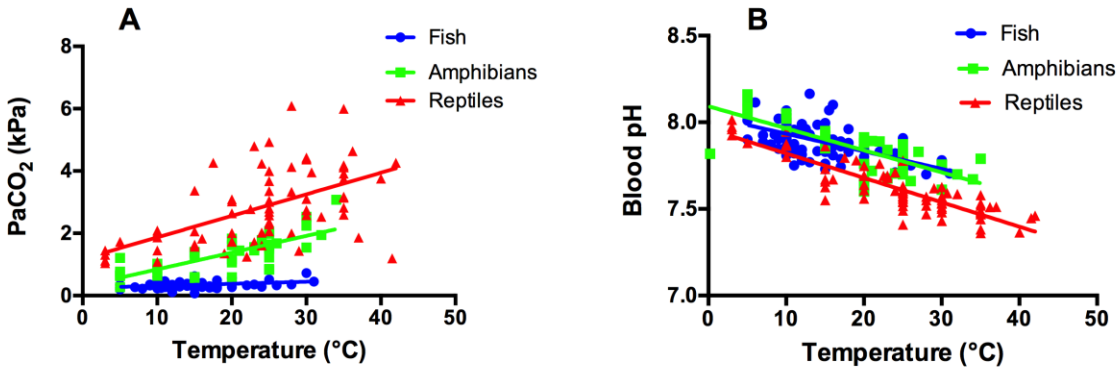

Figure 1. A summary from Ultsch and Jackson, (1996) for fish, amphibians and reptiles for the effects of temperature on **A**) resting $PaCO_2$, and **B**) resting arterial blood pH. Individual symbols are means for between 1-21 studies at that temperature and lines are least square regressions for each class. Symbols are filled circles for fish (n=60 for $PaCO_2$, n = 92 for pHa), filled squares for amphibians (n=40 for $PaCO_2$, n=46 for pHa) and filled triangles for reptiles (n=70 for $PaCO_2$, n=80 for pHa).

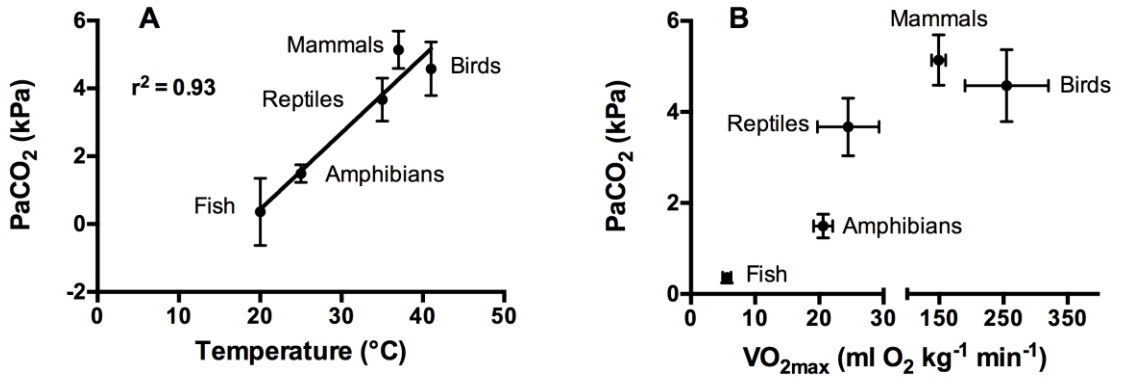

Figure 2. **A**). The effects of temperature on resting $PaCO_2$ (Ultsch and Jackson, 1996) at the temperature where VO_{2max} is greatest for each class of vertebrates (from Hedrick et al. 2015). Values are mean and 95% confidence interval. **B**). The relationship between VO_{2max} at the temperature where it is greatest (from Hedrick et al. 2015) on resting $PaCO_2$ (Ultsch and Jackson 1996). Values are mean and 95% confidence interval. Note the break in the x-axis to accommodate the range of values for the vertebrate classes.