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## Physical calculations of resistance to water loss improve species range models: reply

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Christian et al. (2017) proposed several possible flaws in the methods and logic presented by Riddell et al. (2017) that included potential activity of salamanders during measurements, trimming of the agar model's legs, misinterpretations of the empirical data, limitations on agar models, and the relationship between body size and skin resistance to water loss ( $r_s$ ). We argue that these criticisms are easily addressable, and here, we reinforce our original claim that the agar method for determination of resistance to water loss is flawed. Before responding to these individual critiques, we begin with a deeper criticism of the agar model method and general methodology for determining resistance to water loss that has resulted in the reification of the boundary layer's ecological and physiological importance. Christian et al. also promoted misleading information on the established physical processes for estimating the value of the boundary layer resistance ( $r_b$ ) that relate directly to the flaws of the agar model method. We hope that our response enlightens physiological ecologists on the obstacles that impede the progress of water loss studies.

The complications involving measurements of resistance to water loss arise from its physiological and biophysical components. The total resistance to water loss ( $r_T$ ) is the sum of the resistance of the skin ( $r_s$ ), the physiological component, and the boundary layer of air surrounding the organism ( $r_b$ ), the biophysical component. In a recent study (Riddell et al. 2017), we compared two techniques that are used to decompose the physiological and biophysical components of total resistance to water loss. We compared estimates of  $r_b$  calculated from physical principles to an empirical method that used agar replicas of the focal organism, a terrestrial salamander. The empirical method assumes that agar does not have a  $r_s$ , and therefore, any measurement of resistance is assumed to be the  $r_b$ . Riddell et al. (2017) concluded that the agar

method was an ineffective technique to estimate the boundary layer resistance due to the stark contrast between the empirical and theoretical estimates of  $r_b$  and the violation of physical expectations for both skin and boundary layer resistance. These conclusions have come under scrutiny by Christian et al. due to suspected flaws in methods and logic. Before we address their specific criticisms, we must identify a false conception of the boundary layer promoted by Christian et al. that ignores critical details in the established literature of biophysics.

The boundary layer is arguably the most dynamic, and thus complicated, factor that influences the rate of water loss. The boundary layer refers to the air surrounding an object over which the exchange of heat or mass occurs between the object and the environment (Gates 1980). The processes that influence the thickness of the boundary layer (and thus the resistance to heat and water flux that it provides) change depending upon the prevailing physical conditions. The boundary layer is influenced by the viscosity of the air, the shape and orientation of the object, and the size of the object under all conditions, but the flow rate of the air stream (or wind speed) has a more dynamic relationship with the boundary layer. The flow rate determines the primary force that shapes the boundary layer: inertia from the force of the airstream (i.e., forced convection) or buoyancy from concentration gradients between the animal and the environment (i.e., free convection). During forced convection, the flow rate produces a drag force along the organism that shapes the boundary layer, and  $r_b$  is proportional to and inversely associated with the velocity of the airstream (Gates 1980). As the flow rate decreases, the inertia from the flow rate becomes less influential relative to the buoyant forces, and the primary factor shaping  $r_b$  becomes "differences of air density produced by temperature gradients, by vapor gradients, or by a combination of both" (Monteith and Unsworth 2013). In nature, these convective environments often interact as described in Monteith and Unsworth (2013):

In many natural systems, convection is a very complex process because of continuous changes in windspeed and direction, often coupled with movement of the surface losing heat. During strong gusts, the loss of heat from a leaf or an animal will usually be determined by forced convection, but during lulls free convection may be dominant.

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Much of our knowledge on free and forced convection is described with heat transfer, but as Ernst Schmidt famously discovered, heat and mass transfer are easily related once accounting for differences in momentum (Gates 1980, Monteith and Unsworth 2013). Gates

(1980) also provided quantitative methods involving dimensionless analysis to determine the relative intensity of buoyant to inertial force. These calculations indicate whether the primary physical process shaping the  $r_b$  is forced or free convection. In addition, the established literature provided a general rule-of-thumb of  $0.1 \text{ m}\cdot\text{s}^{-1}$  over which forced convection and under which free convection predominantly shape the boundary layer (Gates 1980). These methods provide a clear, quantitative expectation for determining the convective environment that contributes to the resistance of the boundary layer. These important distinctions were ignored by Christian et al., but these details relate directly to the flaws of the agar model method.

In light of these established dynamics, the comment presented by Christian et al. promoted a false narrative on the processes that shape the boundary layer. Christian et al. challenged the established literature by claiming that free convection is confined to processes within the boundary layer, whereas forced convection shapes the boundary layer. We failed to find specific mention of this distinction in Gates (1980) or Monteith and Unsworth (2013). However, the distinction is irrelevant because it disregards the complex role of the convective environment in estimating the shape and resistance of the boundary layer (as described above). In Riddell et al. (2017), we followed the quantitative methods for determining whether free or forced convection shape  $r_b$ , and the experimental flow rates in Riddell et al. (2017) were lower than the rule-of-thumb threshold under which free convection predominantly shapes the boundary layer. We assume that Christian et al. ignored our efforts in the original publication because they believed that our empirical data supported their narrative. However, the empirical patterns are easily explained by a lack of standardization that has been perpetuated with the agar model method (and measurements of water loss in general) for decades.

Christian et al. suggested that our results in Figs. 2, 3 of Riddell et al. (2017) support the narrative that  $r_b$  increases dramatically under low flow regimes. However, we argue that the patterns observed in Figs. 2, 3 are indicative of a substantial problem in the field of water loss physiology. The patterns between flow rate and  $r_b$  seem intuitive based upon the physical expectations observed under forced convection. However, as we have indicated, the underlying dynamics involved in shaping the boundary layer in our experiments were *free* convection, not *forced*. The increase in  $r_b$  under low flow rates violates our understanding of physics because  $r_b$  should have been independent of the flow rate (i.e., differences in air density shaped the boundary layer). Alternatively, we suggest the cause of these patterns in our study, and in the existing literature to date, occurred due to violating a critical assumption of the equation for resistance to water loss. Resistance to water loss is calculated using:

$$r_T = \frac{\rho}{CWL}$$

where  $r_T$  is the total resistance,  $\rho$  is the water vapor density gradient, and CWL is the cutaneous water loss rate (Feder and Burggren 1992). We argue that water loss studies have likely violated the assumption that the water vapor density gradient ( $\rho$ ) is constant across experimental flow rates (or wind speeds). Reducing the flow rate would decrease the water vapor density gradient as less vapor is flushed out of the chamber thereby decreasing the evaporative demand of the air. Under very slow flow rates, the organism (or agar model) could saturate the chamber thereby reducing  $\rho$  to near zero. As a result, the evaporative demand of the air could be much lower than the study assumes, and physiological studies are more likely to overestimate total resistance to water loss (or  $r_b$  if the agar model technique is being used). A similar process is true for body size. Large individuals are more likely to reduce the water vapor density gradient due to the higher total amount of water vapor contributed to the chamber relative to small body sizes. Therefore, the patterns of  $r_b$  exhibited in Figs. 2, 3 were likely due to the violation of assumptions of the vapor density gradient, not an increase in the boundary layer. We are unaware of any study to validate the vapor density gradient in the chamber, and these errors have had consequences for the narrative on the ecological and physiological significance of  $r_b$ .

The narrative on  $r_b$  suggests that the boundary layer provides substantial resistance to water loss, particularly under low flow rates (Feder and Burggren 1992). Certainly, the boundary layer *can* exert an ecologically relevant resistance to water loss (Riddell et al. 2017), but the lack of standardization to control vapor density gradients have likely resulted in the persistent inflation of the boundary layer's contribution to total resistance. Many foundational studies on water loss have used flow rates below or near the threshold for free convection (Spotila and Berman 1976, Wygoda 1984, Feder and Burggren 1992). These studies are likely to have unintentionally reduced the actual vapor density gradient and proceeded with calculations that inflate estimates of  $r_b$ . As a consequence of an artificially inflated  $r_b$ , studies are more likely to conclude that  $r_i$  is low or near zero. Historically, studies that use the agar method have concluded that some amphibians do not exhibit a skin resistance to water loss (i.e.,  $r_i = 0$ ; Spotila and Berman 1976, Shoemaker and Nagy 1977). However, more recent evidence has dispelled the idea of negligible  $r_i$  in a species of anuran known for a low skin resistance by demonstrating physiological plasticity of water loss rates (Dohm et al. 2001). Therefore, acclimation studies offer a promising avenue for future improvements to the field by refuting the notion that organisms lack a physiological resistance to water loss. Improvements to the general methodology of water loss studies can also resolve the obstacles that impede physiological insight.

Experimental methods for estimating resistance to water loss can be improved by validating that the desired vapor density gradient exists in the chamber. We suggest that chambers contain a vapor pressure meter placed on the

ceiling of the chamber above the animal (and preferably after to ensure the entire animal is exposed to the gradient) to monitor the vapor density of the air. Alternatively, a sub-sample of air from the top of the chamber could be used to validate that the desired gradient exists within the chamber. If the animal saturates the chamber too quickly (due to small chamber sizes or slow flow rates), then the animal will violate the assumed value of  $p$  in the equation by reducing the evaporative demand of the air. The gradient can be manipulated to the desired value by increasing the chamber volume or increasing the flow rate. These steps require validation, but they are common-sense solutions to a method that lacks standardization.

In regards to the specific criticisms by Christian et al., we reject the notion that the agar technique was applied inappropriately due to our efforts to indirectly and directly measure the subject's activity, orientation, and posture during the water loss trials. Flow through systems indirectly detect levels activity based upon the variation in water vapor pressure during the measurement. Spikes or irregular patterns of vapor pressure indicate when an organism is moving (Lighton 2008). We ensured that our measurements occurred during rest by analyzing measurements without spikes or irregularities. Activity was obvious in our study on geographic variation of water loss rates (Riddell and Sears 2015) because the variation in vapor pressure was 18.5 fold higher in active subjects compared to inactive subjects. We also provided animals with sufficient time to acclimate to the experimental chambers. After initially placing the salamanders inside the chambers, we allowed 30 min of time to pass before making any measurements. During this time, we monitored activity during the experiments through a window in the environmental chamber. We found that after exploring their chamber for 10–20 min, they came to rest and assumed a water conserving posture by laying their bodies on top of the hard-wire mesh. The shape and size of the tube also ensured that salamanders rested parallel with the direction of the flow by inhibiting their ability to turn onto themselves. The salamanders would also tuck their legs underneath their body. Therefore, removal of the legs from the agar model was paramount in mimicking the same posture and surface area exposed to the moving air. We ensured that our measurements between agar replicas and the salamanders were similar in posture, exposed surface area, and orientation in addition to monitoring activity.

We agree that Tracy et al. (2007) identified that agar models have limitations, but their temporal threshold is arbitrary and their conclusions are suspect without a control that represents free water. Tracy et al. (2007) based this temporal limitation on a 42 g replica of an anuran, which is more than an order of magnitude the size of our largest specimen. Also, anurans have a much lower surface-area-to-volume ratio than salamanders due to their spherical as opposed to cylindrical body shape. Agar models of smaller organisms with higher surface-area-to-volume ratios will have substantially lower time periods for which they reflect the appropriate conditions to

estimate the boundary layer resistance, if at all. Tracy et al. (2007) also did not provide a control to validate that the plaster or agar replicas exhibited the same rate of water loss as free water. Without such a comparison, their study cannot validate the physical model's efficacy as a surrogate of free water. The assertion by Christian et al. that agar mimics the properties of free water is inherently illogical due to the hydrophilic interactions between the agar and water molecules. Even concentrations of 2% agar solution do not reflect the physical characteristics of free water due to the high number of possible molecular bonds between agar and water (Sterling and Masuzawa 1968). Rather than risk errors due to these unknown limitations, we continue to argue that physical calculations can be less prone to error. Consistent methods will also help improve our knowledge of the physiological processes underlying water loss.

We also argue that flawed methodology has hidden allometric relationships between skin resistance to water loss and body size. Current evidence suggests that body size and  $r_i$  are not correlated in anurans, at least using the agar model approach (Young et al. 2005). Our conclusion on the allometric relationship between  $r_i$  and body mass was based upon underlying morphological factors that likely contribute to  $r_i$ . Vascularization of the skin has been suspected to influence  $r_i$  by determining the "effective thickness" of the physiological barrier to water loss (Lillywhite 2006). As amphibians grow, the per-gram density and length of capillaries diminishes (Czopek 1965) thereby limiting the exchange of gases with their environment in large body sizes (Feder 1988). Also, experimental evidence demonstrates that the amount of fluid delivered to the skin is directly associated with rates of cutaneous water loss (Burggren and Vitalis 2005), and perfusion represents an effective strategy for limiting water loss in salamanders (Brown 1972). Consequently,  $r_i$  might be positively associated with body size due to underlying morphology of blood capillaries. This allometric relationship likely persists within anurans as well because capillary networks in anurans demonstrate the same morphological pattern with body size as salamanders (Czopek 1965). However, the agar model method might have concealed allometric relationships involving  $r_i$  because large agar models have the potential to inflate  $r_b$  by saturating the chamber and thereby reducing the estimate of  $r_i$ . Further scrutiny on the methodology for measuring  $r_i$  will improve our knowledge of amphibian physiology and its ecological role.

The flaws associated with the agar model method promote a false understanding of the mechanisms that contribute to a species' geographic range. In Riddell et al. (2017), we demonstrated the value of mechanistic species range models by identifying the resistance required to maintain habitat suitability. The results from the agar model method suggested that the focal species lacked a physiological capacity to resist water loss and violated the physical expectations of skin resistance. Without a skin resistance to water loss, our approach predicted the entire geographic range of the species to be unsuitable. Only by

including the skin and boundary layer resistance calculated from the theoretical methods could we predict the vast majority of historical captures to be in positive energy balance. The ecological support for our theoretical methods and the consistency of our physiological results with the laws of physics were too strong to ignore. We agree that our approach requires further validation, but we also underscore the critical need to address the flaws in the agar model method that hinder physiological discovery.

In this reply, we elaborated on the flaws of the agar model method from independent lines of evidence that included support from our species range model, the lack of standardization, strong molecular interactions between agar and water, and the violation of established physical expectations for skin and boundary layer resistance. These lines of evidence suggest that the agar model method requires a much more comprehensive evaluation, particularly regarding the discrepancies between empirical and theoretical values. Based on our conclusions, we suggest that ecologists continue to compare theoretical expectations with empirical data to identify inconsistencies that inhibit growth of studies in physiological ecology.

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