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Identifying and Evaluating Patterns in Cardiorespiratory Physiology[1]

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SYNOPSIS. A major theme of many papers in this symposium is the identification of broad physiological trends and patterns that extend beyond the boundaries of data from individual studies. Recognizing patterns in everything from hypoxic ventilatory patterns to regulation of blood gases not only helps the investigator understand specific data sets, but also helps place those data in a broad context. Yet, recognizing physiological patterns is confounded by two factors: phylogenetic relationships and physiological state. Fortunately, the last decade has seen infiltration of sound evolutionary theory, including tools of cladisitic analysis and population genetics, into more and more studies of comparative physiology. However, even when an experimenter carefully accounts for phylogeny, differences in physiological state in the experimental animals can still obscure physiological patterns. Two informal categories of physiological state are described, the first obvious and frequently controlled for, and the second less obvious and typically not controlled for. Examples of the latter, including seasons, rhythms, prandial effects and sex of the animal, are developed to show how ignoring these can lead to considerable misleading variation in cardiorespiratory data sets. Considering physiological state is vital in producing reliable data that can be used meaningfully for delineating broad physiological patterns.

INTRODUCTION

The field of cardiorespiratory physiology continues to thrive, as witnessed by the many illuminating papers presented in this symposium *Comparative Aspects of the Control of Arterial Blood Gases.* One major theme consistent with nearly all of these papers is the identification of physiological patterns, whether they be in breathing activity, regulation of arterial P_{O_2}, or cardiorespiratory responses to internal and external hypoxia. Recognizing such patterns helps the investigator interpret a specific new data set in the context of known, similar physiological processes—*i.e.*, the time-honored mechanistic physiological approach. However, for those interested in doing so, identifying physiological patterns can also help place that data set into a broader comparative context from which we can search for answers to questions such as "What steps were involved in the evolution of this lung ventilatory control system?" and "What are the metabolic costs and benefits of arterial P_{O_2} regulation?"

The intent of this concluding paper in the symposium is to direct the reader to recently developed paradigms for recognizing physiological patterns, and to discuss some important (yet sometimes overlooked), variables associated with physiological state that have obscured our ability to identify these patterns.

THE TRADITION OF RECOGNIZING PHYSIOLOGICAL PATTERNS

Recognizing physiological patterns has long been one of the goals of comparative physiologists. For a comprehensive discussion of this subject, the reader is referred to any of several introspections on the field of comparative physiology (Ross, 1981; Prosser, 1986; Feder *et al.*, 1987; Burggren, 1991). Comparative physiologists often strive to place their data in an evolutionary context, although they have not fully

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availed themselves of the tools of evolutionary biology (Burggren and Bemis, 1990; Burggren, 1991; see major review by Garland and Carter, 1994). Perhaps one of the most pointed criticisms of the traditional paradigms was offered about a decade ago Feder *et al.* (1987). Numerous chapters in that book called into question the major paradigms of comparative physiological research prevalent at that time. Feder (1987) in particular pointed out that, collectively, most studies seemed bent on contributing to a vast data matrix of *all possible adaptations* × *all possible environments*. Although many data existed, few organized attempts had been made to extend observations filling in a single "cell" in this matrix to the broader problem of identifying physiological patterns across the matrix.

Fortunately, the last decade has seen infiltration of sound evolutionary theory, and in particular some of the tools of cladisitic analysis and population genetics, into more and more studies of comparative physiology (see Bennett and Huey, 1990; Martins and Garland, 1991; Garland and Adolph, 1994). Borrowing from the large, and ever-growing data set comprising the *all animals* × *all environments* matrix, the development and application of select and statistically robust analytical procedures for physiological data sets has greatly enhanced our ability to tease apart the literature. It has also aided recognition of patterns leading to a greater understanding of physiological systems and their evolution. In some of the most extensive treatments of the subject to date, Garland and Carter (1994) and Garland and Adolph (1994) discuss the history and present state of evolutionary physiology, and outline in detail analytical paradigms for interpreting physiological data in a phylogenetic framework. As a consequence of the increasing awareness of the power of systematic analysis, we now see comparative physiologists contemplating mapping physiological characters (*e.g.*, CO_2 responsiveness, baroreceptor gain) as primary characteristics in order to understand physiological evolution. Lauder (1991), for example, advocates construction of cladograms based on cost of locomotion and fuel mobilization efficiencies. The use of such a paradigm—drawing upon biochemical and physiological characters rather than morphological ones—was almost unimaginable a few short years ago. The power of using non-morphological traits is only just beginning to be realized in systematic analyses as well. For example, the interpretation and evolution of the endothermic condition in tunas varies starkly depending upon whether one employs morphological or molecular phylogenies (Finnerty and Block, 1995).

Variation and Pattern Recognition

Even if an experimenter carefully accounts for phylogeny, recognizing physiological patterns will still be confounded by non-phylogenetic related variation in the data set. Bennett (1987) and Feder (1987) have extolled the virtues of a data set containing variation. Pointing out what he called "the tyranny of the Golden Mean," Bennett (1987) suggested to us that comparative physiologists, in their efforts to produce a single average number describing a physiological condition, are too quick to overlook (or ignore) outliers in their data. In fact, the outliers often constitute useful, if not the most useful, data from which to refine our understanding of physiological patterns.

Why do apparent outliers create variation in the aggregate data set? Variation in physiological data arises for at least two major reasons—variation in physiological state and the use of different techniques. Once again, we can return to the concept of a data matrix. In this case, consider a data matrix of *all possible physiological states* × *all possible techniques*. I will defer on discussion of *all possible techniques*. Most of us have had the experience of slightly altering a measurement technique, or a way of preparing an experiment, only to find quantitative and sometimes qualitative changes in the resultant data we collect! Variation derived by differences in technique can be minimized, but not eliminated. The other major source of variation—*i.e.*, outliers that depart from the expected mean—in physiological experiments is the result of the physiological state of the animals, and is a "real" and important source of genetic- or

environment-induced variation deserving analysis (Bennett, 1987).

PHYSIOLOGICAL STATE AS A SOURCE OF VARIATION AND PATTERN

The control of physiological state is a hallmark of good physiological investigation. Most comparative physiologists either directly recognize the importance of physiological state of their animals to the outcome of their experiments, or have been trained to minimize variation from physiological state without consciously thinking of why they are doing so. Nutritional state, for example, falls in a category of physiological state that is often readily observed and can be monitored during the course of the experiment. Indeed, in a survey that I have made of recent comparative physiological papers drawn at random from two prominent journals in the field, 80% of the studies report the feeding regimes under which animals have been held prior to experimentation. Additional examples of physiological state that are frequently controlled include developmental state (embryo, larva, adult), level of activity (resting, active, sleeping) and respiratory state (breathing, breathholding). Numerous studies of cardiorespiratory physiology, including some outlined in this symposium, have not only controlled for these physiological states, but have actually used changes in states as experimental variables.

Unfortunately, design of control in physiological studies do not seem not to have kept pace with the rate at which comparative physiologists have discovered factors affecting physiological state. For example, we now know that an animal's sex may affect its heart rate variability (Altimiras *et al.*, 1996) and that hormones associated with mammalian and reptilian pregnancy directly alter both ventilation (Hannhart *et al.*, 1990) and the Hb-oxygen equilibrium curve (Ragsdale and Ingermann, 1991). Yet, how often do we even bother to determine the gender and reproductive state of the animals we investigate, let alone report these variables (see Example #3, below)? Indeed, it is highly ironic that, even as comparative physiologists are becoming much more rigorous in controlling and correcting for phylogeny in our experimentation, we may be falling behind in our efforts to adequately control physiological state.

To emphasize the often profound effects that some of these infrequently controlled physiological states can have on cardiorespiratory data, consider four examples dealing with prandial state, season, sex of the animal, and circadian rhythms.

Example #1—Prandial effects.—As indicated above, about 80% of comparative physiological studies have controlled in part for prandial state, either by working on animals allowed to feed freely (or frequently) or by working on animals fasting for some usually predetermined period. (Interestingly, this knowledge of the effects of nutritional state on physiological state is rarely used as a tool to probe actively the physiological system in question). However, among this large percentage of physiologists who specify a particular nutritional state, about 1/2 let their animals feed *ad libitum* until experimentation, while the others enforce a fast upon them!

The effects of feeding and fasting on metabolism and related physiological variables such as heart rate and cardiac output have been widely documented in both invertebrates and vertebrates (see Burggren *et al.*, 1993; Wang *et al.*, 1995). Known as Specific Dynamic Action (SDA), the ingestion of food causes increased metabolism and cardiac output that seem to follow a time course that depends on both the amount of food ingested and the species. A study of the time course of SDA in three terrestrial/intertidal crabs showed relatively little intraspecific variation, but great interspecific variation (Burggren *et al.*, 1993). *Cardisoma guanhumi* and *Ocypode quadrata* showed a profound SDA response, while no significant response was detected in *Goniopsis cruentata* treated identically before, during and after the same feeding protocol.

One of the most interesting accompaniments to SDA is the so-called "alkaline tide," which has been documented in animals ranging from alligators to humans (see Wang *et al.*, 1995). The ingestion of a large meal often results in a rise in plasma pH, as H^+ is actively transported into the gut to

aid in the digestive process. Alkaline tide presents a real paradox, in that even as plasma pH rises (and by convention the central stimulus for ventilation falls), ventilation actually rises to support the SDA effect on metabolism. This suggests the possibility of a different ventilatory control mode in prandial and post-prandial states. In the same way that our knowledge of physiology may be largely grounded in daytime responses, so too our knowledge of ventilatory control and acid-base balance may be grounded largely in fasting rather than in actively digesting animals.

Clearly, interspecific comparisons attempting to discern physiological patterns could be seriously compounded by the variable influences of SDA unless they are carefully controlled. Is a ". . . fasting animal . . ." used in an experiment actually in the declining phase of metabolism following a meal (which can last for days in some species) or is it in a near steady-state in a post-SDA phase? Clearly, there is no single "correct" nutritional state appropriate for all experiments, but knowledge of how nutritional state can influence physiological processes can help in reducing unwanted variation and in recognizing physiological patterns.

Example #2—Seasonal effects.—Of the comparative physiological papers that I surveyed, only 28% reported the season (time of year) in which animals were collected and/or experimented upon. Even then, all too often experimenters, especially those working in temperate climates where large seasonal climatic variations occur, assume that "season" is primarily a temperature effect to be reckoned with in their experiments. Whereas winter animals in temperate climates may indeed experience much lower temperatures than in summer, seasonal effects involve far more than just temperature. Changes in food availability and day length are but two of several important environmental changes that can lead to adjustments in nutritional state, energy reserves and hormonal balance as a function of season. The common pond fish, the bluegill, will thrive at winter temperatures of 6–8°C *if* the day length is about 8–10 hr—a light level appropriate for a winter temperature. However, these same fish will weaken and even die if day length is increased to summer levels of 12–14 hr with no other change in environmental variables (J. Roberts, personal communication). In this context, my survey of comparative physiological studies showed that 44% of experimenters reported information on the light:dark cycle in which their animals were maintained. Only about 10% of those experimenters employed a "natural" light day cycle, with the remaining 82% of those who reported a light:dark cycle apparently arbitrarily picking a 12light:12dark cycle. Only 8% of all studies surveyed *concurrently* presented data on light:dark cycle and time of the year so that the reader could assess the appropriateness of the lighting regime.

Cardiovascular physiology provides a fascinating lesson in the impact of seasonal (and circadian) effects. Otto Loewi shared the 1936 Nobel Prize with Henry Dale for discoveries involving neurotransmission. Loewi's key experiment involved stimulating the vagus nerve of a frog until its heart stopped. He then withdrew blood, containing traces of acetylcholine (ACh) released from the stimulated vagus nerve, from that heart and injected the blood into the heart of a second frog. The second frog's heart promptly stopped. In this elegantly simple experiment, Loewi demonstrated that a neurotransmitter had been released from the end of a stimulated vagus nerve. As the story goes, Loewi performed the very first of experiments at three in the morning on the eve of Easter Sunday. Loewi was not only brilliant, but lucky, for the ACh sensitivity of the heart of the frog is highest in spring and in the early morning hours. If Loewi had performed that key experiment in the afternoon of an autumn day, he may not have had positive results, for the ACh sensitivity of the frog heart is lowest in the autumn and in the afternoon! Seasonal effects unrelated to temperature have also been found in the hemodynamics of amphibians, where the priming and pumping roles of the atria, ventricle and conus of bullfrog larvae vary between spring/summer and fall/winter (Pelster and Burg-

gren, 1991). Clearly, season affects physiological state, and a lack of careful control for this effect may contribute to variation in data and obscure recognition of cardiorespiratory patterns and relationships.

Example #3—Sex effects.—Controlling for the sex of animals used in experiments ranges from being a trivial matter to a nearly impossible task, depending on the species used. Determining the sex of many types of birds and fishes is straightforward, but determining the sex of some types of reptiles requires dissection or at least laparoscopy to examine internal organs! Perhaps this is why only 16% of comparative physiological studies in my survey actually reported the sex of their animals.

The importance of the sex of the animals to an experiment depends in part upon the nature of the experiment and the subtlety of the experimental perturbation. Intuitively, sex might be expected to have little effect on the interpretation of experiments looking at the effects of large temperature changes on plasma pH, or arterial chemoreceptor responses to arterial blood gases induced by hypoxia or hypercarbia. Yet, the sex of the animal being studied has profound effects on, for example, heart rate, heart rate variability, and atrial inotropic responses in fishes (Davie and Thorarensen, 1995; Altimiras *et al.*, 1996), the sensitivity of vascular smooth muscle to various vasoactive drugs in mammals (Shechtman and Katovich, 1993), and the metabolism of lizards (Garland and Else, 1987). As already mentioned, hormones associated with pregnancy have also been implicated as agents of change for respiration and blood gas transport (Hannhart *et al.*, 1990; Ragsdale and Ingermann, 1991). Given our increasing understanding of the broad-ranging effects of androgens and estrogens, such findings should hardly be surprising.

Example #4—Circadian effects.—To what extent does our knowledge of comparative physiology reflect what animals do between the typical experimenter's breakfast and dinner? To what extent does the *variation* in extant physiological data reflect the subset of data collected in the evening or night-time hours (primarily by graduate students and post-docs!)? And to what extent have all of us missed a significant physiological finding by not keeping the same early morning hours as Otto Loewi? We can be led seriously astray if we ignore circadian effects on physiological state. Consider, for example, the oxygen consumption of honeybees, *Apis mellifera*, measured in constant darkness over a three day period (Stussi, 1972). Up to a 30-fold difference in metabolic rate occurs between day and night! While this represents an extreme example, this phenomenon occurs to a significant degree in many other invertebrates. Large daily changes in metabolism are also evident in many vertebrates (see Hastings *et al.*, 1991). In some cases, these changes are due to phenomena such as torpor in endotherms, but many other ectotherms and endotherms with little or no daily rhythm in body temperature still show profound circadian rhythms in metabolism. Importantly, such changes in metabolic rate must reflect changes in cardiac output, blood gases, and the many other interrelated variables that support tissue metabolism.

Despite this critical importance of circadian rhythms on respiratory and cardiovascular physiology, my survey indicates that only 4% of investigators report the actual time of day during which experiments were performed. Moreover, as indicated in example #2 above, few experimenters apparently control the lighting regime, even though light cycles are among the most powerful of zeitgebers for circadian rhythms.

More than just controlling for time of day, incorporating it into the experimental design may be very useful in determining patterns in cardiorespiratory physiology. An excellent example of this is Dumsday's (1990) study of the heart rate of the toad *Bufo marinus*, which at the outset was designed to examine variation in heart rate and its causes. By using continuous, long-term recording, he was able to determine the often profound effects not only of time of day, but also of lighting conditions, nutritional state, etc. Certainly, the advent of telemetry and computerized data recording is making long-term, around-the-clock recording of

physiological data much easier than in the past. Such approaches will go a long way towards identifying the effects of circadian rhythms and helping to identify patterns and sources of variability in physiological data.

CONCLUSIONS

This brief essay, and particularly the four examples immediately above, highlight the importance of considering physiological state in producing reliable data and then meaningfully interpreting these data as evidence of broad physiological patterns. Assuming that the data have been collected in a technically robust fashion, all data sets must be viewed as "correct," even if we do not anticipate the findings and the degree of variation is large and conformation to known patterns is small. In many instances, this variation "merely" reflects differences in physiological states, which as we have seen in the examples above, have broadly ranging effects (*e.g.*, those on cardiorespiratory physiology, including arterial gases). Accordingly, the potentially large impact of physiological states only infrequently controlled for must be recognized in the design of physiological experiments.

Yet, the goal of cardiorespiratory physiologists should not always (or even frequently) be to eliminate variation in data by controlling for physiological state. As evolutionary biologists have demonstrated repeatedly, variation in data is a rich source of information that helps to understand the larger patterns in the systems we study. Accordingly, cardiorespiratory physiologists may profit from designing experiments that introduce changes in physiological state with the specific purpose of elucidating the myriad of subtle and not-so-subtle factors that influence variables like blood Po_2, intracellular pH, or cardiac output. In this respect, setting up experiments with conflicting and complementing physiological states may be very helpful in delineating physiological patterns. To draw upon Example #1 from above, what happens if an animal experiencing an increased plasma pH because of an alkaline tide following a large meal is exposed to hypercapnia or even infused with acid? Will the offsetting decline in plasma pH cause an increase, decrease or no change in ventilation (all of which at this time could be argued as possible)? Routinely placing physiological systems on the horns of an experimental dilemma, rather than pushing it in a single direction in which we already know it will move, will be an increasingly rewarding component of experimental design in cardiorespiratory experiments.

Similarly, determining the variation in our data that results from season and rhythms will also greatly help us define and understand emerging patterns in cardiorespiratory physiology. I strongly recommend this experimental approach knowing full well that it means for many of us both early mornings and late nights in the laboratory. Recommending experiments that embrace season as a variable is even more perilous, for as a mode of physiological experimentation it flies in the face of the traditional time course of both extramural granting agencies and the reward system under which most of us labor. Yet, our own serendipitous consideration of season as a confounding variable actually enormously clarified a data set that on preliminary examination had appeared quite chaotic (Pelster and Burggren, 1991).

Comparative physiologists have long sought to place their data in a broader context—to fill in new cells in the large matrix of *adaptations* × *environment* described at the outset. Continuing to fill in additional cells remains important, as it is from this information-rich background that new syntheses are made. Heretofore unrecognized patterns and interactions can be identified as a direct result of employing techniques that pivot on systematics and physiological state. In some cases, experimenters must retool with new statistical procedures and experimental techniques. In many cases, however, it is a matter of more effort in experimental design, with the large reward of more robust data sets that point with clarity to major physiological patterns.

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