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UNITY IN DIVERSITY: A Perspective on the Methods, Contributions, and Future of Comparative Physiology

George N. Somero
Hopkins Marine Station, Stanford University, Pacific Grove, California 93950; e-mail: somero@leland.stanford.edu

This brief essay on the methods, objectives, achievements, and future promise of the discipline known as comparative physiology focuses on three principle issues. First, how is this discipline defined in terms of its approaches and goals? What does the adjective comparative denote, and what makes the comparative approach unique? Second, what are illustrative examples of the successes of the comparative method in the study of physiology? Why has the comparative approach so often been critical in the development of basic understanding of physiological systems? Third, how is comparative physiology likely to contribute in the near future to the biological sciences, here broadly defined to include research ranging from study of the consequences of global change to the development of biomedical technology? And, conversely, how are advances in other disciplines in biology likely to enhance comparative physiology?

I hope to demonstrate that comparative physiology is an essential complement to other disciplines within physiology that commonly exploit a relatively small number of so-called model organisms in attempts to elucidate basic mechanisms of physiological function. I argue that there exists a creative interplay between physiologists doing comparative work and others who carry out primarily reductionist studies with model species. Whereas the latter types of studies offer the comparative physiologist many useful new techniques and insights into basic mechanisms, it is the comparative physiologist who often uncovers important new phenomena for investigation and who, through the logic of comparative analysis, elucidates key principles that might not emerge from the study of conventional model organisms.

THE COMPARATIVE APPROACH: UNIQUE ATTRIBUTES

What, then, is comparative physiology, and—importantly in this age of biomedically oriented work with model systems—why should one do it? To use Knut Schmidt-Nielsen's apt phrase, comparative physiologists are fundamentally curious about "how animals work." To approach this central issue, comparative physiologists typically study variation among organisms in commons types of

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physiological systems in an effort to determine how this natural variation allows organisms to function in the diverse habitat conditions they face. This physiological variation may be interspecific and acquired over many generations, or it may be variation that occurs during the life span of the individual. The latter might occur through field acclimatization to new conditions, as might be linked to seasonal changes in the environment, or in controlled studies of laboratory acclimation, in which only a single factor is manipulated.

The success of the comparative approach is marked by the emergence of a unity of principle from the study of diverse solutions to a common problem, hence the theme of "unity in diversity" that characterizes the basic approach of comparative physiology. This underlying logic of the method of comparative analysis often reveals those aspects of the process in question that are invariably conserved, either through evolutionary change or through regulatory processes occurring in the individual in response to a physiological challenge. These conserved characteristics of the organisms' physiology tell us what is most important about the process in question and provide lessons for how organisms are modified during evolution, acclimatization, and acclimation to allow sustained activity in the face of changes in the environment.

The history of comparative physiology reveals that natural variation in physiological systems has, in fact, played two important and somewhat distinct roles in the development of the discipline. Natural variation has not only been at the intellectual core of the field, but has also been the focus of an exploratory spirit in comparative physiologists, the best of whom seem to be natural historians at heart. As is evidenced in the writings of many of the century's pre-eminent comparative physiologists, for instance, George Bartholomew, Peter Hochachka, August Krogh, C Ladd Prosser, Knut Schmidt-Nielsen, Per Scholander, and C Richard Taylor, fascination with natural variation has sparked a tremendous amount of exploratory effort—with the word exploratory denoting not only intellectual exploration in the laboratory but also adventurous field exploration in out-of-the-way corners of the planet [read Scholander et al's classic paper (1) on freezing resistance in Labrador fishes for a taste of adventurous physiology and a sense of the colorful nature of writing formerly allowed in the now rather staid scientific literature!]. (Parenthetically, I've long been curious as to what early influences lead one to pursue this type of comparative-exploratory work. Perhaps it is close childhood contact with pristine environments replete with diverse assemblages of species that draws comparative physiologists toward their particular calling. Conversely, it may be that growing up within the confines of a large city draws one to work with models such as rodents, flies, and *Escherichia coli.*)

The charting of natural variation by comparative physiologists has uncovered a wealth of novel phenomena begging deeper, mechanistic analysis. Analyses of these natural curiosities—for instance, Why don't polar fishes freeze? How can cells withstand desiccation? Why can desert rodents survive without drinking water? Why do sharks accumulate urea? What allows prolonged breath-hold diving in seals? What keeps tuna fishes warm?—by means of the comparative

method have often led to major new concepts about the most basic aspects of physiology.

SUCCESS OF THE COMPARATIVE APPROACH: ILLUSTRATIVE EXAMPLES

There are many examples of the successes of the comparative approach in deducing unifying principles through study of physiological diversity. Below, I focus on two illustrative cases that I think are especially relevant for an essay designed to convey an historical perspective on the field of comparative physiology. Both examples concern extensions of a core concept in physiology credited to the great 19th century physiologist Claude Bernard, the conservation of the *milieu intérieur.*

The first case involves what can be regarded as the smallest constituent of the *milieu intérieur,* the proton, and focuses on the development of our understanding of the ultimate cause of the variation in pH observed among organisms with different body temperatures [see the excellent reviews by Reeves (2) and Cameron (3) for details]. During the early decades of study of physiological pH values, emphasis was strongly focused on the pH of blood of mammals with core temperatures near 37°C. Thus arose the concept that the "normal" pH of organisms was near 7.4 (2). This paradigmatic view of the normal pH of cells was strongly held for decades, despite the gradual accumulation of data, beginning as early as the late 1920s (4), showing that the pH values of blood of ectothermic organisms whose body temperatures were lower than 37°C were typically a few tenths of a pH unit above 7.4. It was not until the early 1960s that physiologists, spurred by work of Eugene Robin (5) and others, took a renewed interest in temperature-pH issues. As new information was gathered on the pH values of diverse ectothermic species, it became increasingly clear that the mammalian paradigm was correct only in a limited sense, that is, only for organisms with body temperatures near 37°C. Thus arose the need for a new unifying principle that could explain the ultimate cause of temperature-dependent variation in pH. The alphastat hypothesis (2), which was based largely on theoretical and experimental efforts of Herman Rahn, Robert Blake Reeves, and their colleagues, provided the needed synthesis. The alphastat hypothesis provided a precise mechanistic account of why it is important to conserve pH, not at a constant value at all temperatures, but at a value that favors a stable fractional dissociation state of histidine imidazole sidechains. As Cameron (3) points out in his review, there were important historical precedents for the alphastat hypothesis, yet it took the clear statements of Rahn, Reeves, and colleagues and the large comparative data base accumulated on pH values of organisms with widely different body temperatures to establish the new paradigm. The alphastat hypothesis provided biochemists with a clearer sense of why changes in pH affect so many aspects of protein function, and why the preservation of protein structure and function at different temperatures demands a temperature-dependent solution pH. The comparative approach thus was cor-

rective of an erroneous view held by the majority of physiologists for decades and proved to be critical in identifying a new unifying principle of physiology. With a deeper understanding of temperature-pH relationships both biomedical practice and in vitro biochemistry could be conducted in a more rational and realistic fashion.

A second illustration of how the comparative approach enabled a unifying theory of physiology to be developed from a broad examination of physiological diversity concerns another key aspect of the composition of the *milieu intérieur*, the low-molecular-weight organic molecules (organic osmolytes) that constitute the bulk of osmotically active materials in cells of osmotically concentrated species. Comparative physiologists surveying the composition of the cytosol of widely different taxa—including animals, plants, protists, fungi, and bacteria—discovered that four distinct groups of low-molecular-weight organic molecules were exploited in osmotically concentrated organisms: free amino acids (e.g. glutamate, alanine, and proline) and their derivatives (e.g. taurine), methylammonium and methylsulfonium solutes (e.g. trimethylamine-*N*-oxide and glycine betaine), polyhydric alcohols (e.g. glycerol and trehalose), and urea (6). Was there something special about these particular types of organic solutes that made them fit for use at high concentrations in osmotically concentrated organisms? What unifying principle could explain the recurrent exploitation of these particular types of solutes by phylogenetically diverse organisms? The unity underlying this diversity in organic osmolyte composition was first explained by Mary Clark (7), who drew attention to the findings that Hofmeister made in the 1880s regarding the differential effects of various ions on protein solubility. His experiments led to the development of an empirical ranking of ions (the Hofmeister series) in terms of their influences on protein stability and solubility. Clark pointed out that the chemical groups found on organic osmolytes typically resemble stabilizing organic ions (e.g. methylammonium ions) of the Hofmeister series. She hypothesized that natural selection favors the accumulation of organic molecules whose fitness results from their favorable effects on protein structure and function, with urea being a curious exception to this rule, as discussed below. This hypothesis was a major extension of Bernard's concept concerning the importance of regulating the *milieu intérieur.* Regulation of the organic osmolyte composition of the cytosol is recognized to be important not only in the short-term responses of euryhaline osmoconformers but, even more fundamentally, in the initial development at the dawn of cellular evolution of an intracellular solution that was compatible with macromolecular function.

The principles underlying osmolyte fitness for macromolecular function and structure have received detailed analysis from physical chemists, biomedical researchers, and, most recently, biotechnologists. The physical chemical studies of Serge Timasheff (8), Wayne Bolen (9), and their colleagues have provided an elegant mechanistic explanation of how organic osmolytes affect protein structure. Timasheff, who had made seminal discoveries about the effects of inorganic ions on proteins, became fascinated with the physicochemical basis for evolutionary

selection of particular classes of organic osmolytes. His studies of organic osmolytes showed that, with the exception of urea, they are excluded from the water immediately adjacent to the protein surface. This preferential exclusion of stabilizing osmolytes favors a compact folding of the protein, whereas the preferential interaction with the protein of destabilizing solutes like urea favors unfolding (denaturation). Bolen and colleagues (9) recently demonstrated that these solute effects are dominated by the influences that stabilizing and destabilizing osmolytes have on the solubility of peptide backbone linkages; side-chain effects are of much less importance. Thus, in common with the comparative analysis of pH-temperature relationships discussed above, the comparative study of organic osmolytes has led to a deeper understanding of how proteins are stabilized within the cell and, thereby, to an appreciation of a fundamental principle underlying the evolution and regulation of the *milieu intérieur.*

Comparative studies of organic osmolyte systems have also had important impacts in biomedicine and biotechnology. The discovery by Paul Yancey (6) that methylammonium compounds present in urea-rich fishes counteract the effects of urea on proteins led to a re-examination of the intracellular milieu of mammalian kidney cells, most notably, by Maurice Burg and colleagues (10). The heretofore unexplained occurrence of methylammonium solutes like glycerophosphorylcholine in cells of the inner medulla of the kidney could now be interpreted as an example of urea-counteraction. Homer Smith, whose classic book *From Fish to Philosopher* (11) discussed the anomaly of high urea levels in cartilaginous fishes, would no doubt be pleased to learn that sharks and philosophers (at least in the latter's inner medullas) opt for a common osmotic strategy.

In biotechnological research designed to develop solution conditions favoring stability of macromolecules, stabilizing osmolytes are playing an increasingly important role. What is termed "formulation" science is focusing strongly on the lessons provided by comparative physiologists, whose studies of natural variation in osmolyte systems point the way toward engineering media for preservation of biological materials in both the frozen and the dried states.

In summary, the two lines of research discussed above, each of which focuses on a different aspect of the evolution and regulation of the *milieu intérieur,* illustrate how comparative study of physiological diversity can lead to discovery of unifying principles of biological design. In many such cases, it is difficult to imagine how substantial progress in developing these key principles could have been made without the comparative approach.

HOW PHYSIOLOGICAL SYSTEMS GET TO BE THE WAY THEY ARE: EVOLUTIONARY PHYSIOLOGY

Much as physiologists are interested in how organisms work, they are increasingly interested in how physiological systems arose initially and how they achieved their particular states in organisms living under different environmental condi-

tions. Origin and adaptation are central themes throughout comparative physiology. This being said, providing definitive evidence for the adaptive significance of trait, much less explaining how the trait came into being (or, for that matter, even defining what a trait is), is often a difficult and controversial matter. There has been much uncritical writing about the putative adaptive significance of traits, and the tradition here is a long one. Thus, prior to the 1859 publication of Charles Darwin's *The Origin of Species by Means of Natural Selection,* many leading scholars in the natural sciences viewed the traits of plants and animals as illustrations of God's ability to create perfectly adapted organisms [see the fascinating account of the adaptationist thinking of Linnaeus, Buffon, Cuvier, Lamarck, and other predecessors of Charles Darwin in historian John Greene's book *The Death of Adam* (12)]. The so-called Panglossian Paradigm (13) did not originate with 20th century biologists. The last two decades have been marked by a serious reexamination of the more secular concept of adaptation, with the seminal paper by Steven J Gould and Richard Lewontin (13) providing much of the stimulation for this analysis. Although this brief essay does not permit an extended discourse on the shortcomings of the adaptationist paradigm (for a lengthy analysis see Reference 14), two clear lessons have emerged from the critique of studies of physiological adaptation. One, as outlined clearly by Gould and Lewontin (13) and others (14) is that caution must be used in employing terminology pertaining to adaptation. A trait serving some function in a contemporary species should be viewed as an aptation, rather than as an adaptation, unless one can demonstrate that the trait currently fulfills the function for which it was originally selected. This logical vocabulary for discussing the function of traits has not been widely adopted by physiologists, for reasons that are not entirely clear. Perhaps in the new millennium, things will be different.

Adaptationist thinking is currently undergoing analysis that leads to a second and related lesson concerning the difficulties of discerning unambiguously if a particular characteristic of an organism represents an adaptation (or aptation) to an environmental condition or, instead, is merely a reflection of phylogeny (phylogenetic inertia or historical contingency) (15). Study of putative adaptive variation should be conducted in the appropriate phylogenetic context, in order to be what is commonly termed a phylogenetically correct analysis. Thus, the study of physiological evolution and adaptation is now increasingly being conducted jointly with phylogenetic analysis, the latter often employing molecular techniques. This type of dual analysis is proving to be an effective means for discerning the adaptive importance of physiological variation.

COMPARATIVE PHYSIOLOGY IN THE SERVICE OF ECOLOGY

The study of adaptive variation among species not only can provide insights into basic physiological principles—the unity that emerges from the study of diversity—but can also yield rewards for investigations at higher levels of biological

organization. The discipline of ecological physiology, which builds on the efforts of comparative physiology, is concerned with how changes in the environment are apt to modify ecosystems through direct and indirect effects of the environment on the functions of organisms. As comparative physiologists clarify more definitively what the environmental optima and tolerance limits are for diverse physiological processes, the ability of ecologically oriented physiologists to make predictions about the effects of habitat change will be enhanced. For instance, characterizing the differences between eurythermal and stenothermal species and discovering the physiological differences that explain their varied thermal tolerance limits may assist ecologists in developing predictions about the effects of global warming.

GENETIC BASES OF PHYSIOLOGICAL VARIATION

The genetic bases of physiological variation are becoming an increasingly important focal point in comparative physiology. This focus is certain to strengthen the discipline for several reasons. As the genetic underpinnings of physiological variation are discovered, ambiguities about the adaptive significance of traits and the origins of these traits during evolution will be reduced or eliminated. An illustration of progress along these lines is provided by recent studies of the genes encoding antifreeze glycoproteins (AFGPs) in polar fishes (16, 17). The presence of AFGPs with the same primary structures in both Antarctic notothenioid fishes and Arctic fishes of the cod family might conceivably be a consequence of the occurrence of a single progenitor AFGP-encoding gene in a common ancestor of these two lines of fishes. Such an ancestral AFPG gene might have persisted in the two lineages during their diversification and have taken on a key role in cold adaptation when the two groups independently encountered the threat of ice formation in their body fluids. However, as Chen et al (16, 17) have shown, the genes encoding the AFGPs of Antarctic and Arctic freeze-resistant fishes arose independently in response to cooling of their habitats approximately 14 and 2.5 million years ago, respectively. Moreover, Chen et al showed that the AFGP-encoding gene of notothenioid fishes arose from portions of a gene, including fragments of an intron region, that encoded the proteolytic enzyme trypsinogen (16). The progenitor of the AFGP-encoding gene of Arctic cods is unknown (17). Studies of AFGPs and antifreeze peptides demonstrate that adaptation to the threat of freezing has led to the independent origin of macromolecular antifreezes in numerous taxa and that the genetic raw material used to effect these adaptations has been highly varied.

Analysis of how these diverse macromolecular antifreezes work has revealed a unity of mechanism among structurally diverse antifreeze molecules. Despite wide variations in primary, secondary, and tertiary structure, all antifreezes studied to date appear to have surfaces that interact strongly with ice, leading to inhibition of ice crystal expansion (18). Here again, the comparative approach has elucidated

a fascinating example of convergent evolution toward a unified mechanism of function in disparate lineages.

I find it satisfying that the initial curiosity that drove Scholander and his colleagues to perform adventurous field studies in the Arctic has led, some four decades later, to the discovery of how the genes enabling fishes to resist freezing originated, to elucidation of how the AFGPs and peptides work at the molecular level, and, recently, to investigations of how these novel molecules might be exploited through biotechnology to preserve cells at low temperatures. Here is a clear illustration of how the comparative approach has contributed broadly to the biological sciences.

FUTURE PROSPECTS FOR COMPARATIVE PHYSIOLOGY

It is a cliché, although nonetheless usually a partial truth, to say that the future of a field of research will be technology-driven. The validity of this statement in the context of comparative physiology is seen in on-going exploitation of a variety of new methodologies that are allowing novel experimentation across a wide range of spatial and temporal scales and with organisms whose study has been difficult or impossible to carry out in the past. Field work using remote sensing technology based on miniaturized, microprocessor-based systems and satellite-based communication links is allowing heretofore difficult-to-monitor organisms to be tracked over long distances and periods of time in their natural environments. For instance, current work on large pelagic fishes like bluefin tuna, which move through thousands of kilometers of the ocean and change depth frequently, exemplifies the potential of these new technologies to advance field-oriented physiological studies (19). Through learning how difficult-to-monitor organisms behave in the field, the physiological characteristics of these organisms will be placed in a more meaningful context and, as is almost certain to be the case in such natural history explorations, new phenomena will be discovered that will lead to novel lines of study.

Molecular approaches are already playing major roles in comparative physiology in a wide variety of contexts, and the power of this new technology will continue to contribute to the development of the field in many ways. Studies in which specific proteins of animals are modified using site-directed mutagenesis and then expressed in vivo, where the physiological impacts of the mutation can be studied, are beginning to shed new light on such processes as muscle function. Recent work on molecular engineering of regulatory subunits of myosin in *Drosophila* is illustrative of the power of this approach (20, 21). Genes removed from or introduced into organisms, followed by physiological experimentation designed to test the effects of these manipulations of the genome, will help to more fully define the roles of genes whose products are of putative importance

in adaptation. An interesting illustration of the genetic knockout approach is given by a recent study in which the myoglobin gene was knocked out of the mouse (22). As more and more species become amenable to these types of molecular genetic manipulations, comparative physiologists will be increasingly able to test novel predictions about adaptive significance of traits. Comparative analysis of protein sequences is revealing the regions within proteins where adaptive change appears to be focused, and how amino acid substitutions at these adaptational hot spots bring about changes in function (23). Rapidly developing techniques for screening patterns of gene expression, for instance, the DNA microarray methodologies that involve robotics and permit huge data sets to be gathered, are likely to contribute to our understanding of environmental and developmental regulation of gene expression (24). In one sense, the survey studies made possible by DNA microarray technology are logically equivalent to a natural history survey, albeit at the fine-scale of molecular diversity. As Brown & Botstein (24) state in a recent review, the vast numbers of new genes being discovered in genome sequencing efforts are "an exhilarating reminder that much of the natural world remains to be explored at the molecular level." Once physiologists determine what genes are turned on, in different tissues, under different types of environmental stresses, then the functions of these genes may become clearer (or, in many cases described for the first time) (24), and unifying new concepts about gene expression in response to environmental change may emerge.

To imply that the future success of comparative physiology will depend entirely on the exploitation of molecular methods and other new types of technology is not my intent, for such a prediction would ignore an essential point: the strength of the discipline of comparative physiology lies not with any particular method—these evolve rapidly and no one can predict what, ten or twenty years down the road, will be the leading-edge method of the day—but rather with a unique logic for resolving biological questions. The logic of comparative physiology permits analysis of a wide range of questions, ranging from the determination of basic mechanisms of physiological function to the elucidation of evolutionary pathways to the prediction of effects resulting from environmental change. Common to these diverse goals is what is probably the most famous principle of comparative physiology, the August Krogh principle (25). As commonly phrased, this principle states that, for any particular question in biology, nature holds an ideal study system. It is up to the creativity of the biologist to identify this system to advance the study in question. Although comparative physiologists may tend to view the August Krogh principle as their own, this principle has been applied throughout biology, for instance, in the choice of tractable organisms for studying genetics and heredity (first *Drosophila*, then simpler systems like yeasts and bacteria). By judicious application of the August Krogh principle, comparative physiologists have been able to select appropriate sets of organisms with which to conduct their analyses. Much of the discussion of phylogenetic correctness in experimental design can be viewed as an attempt to develop more logical means for exploiting the wisdom found in the August Krogh principle!

With the appropriate choice of study organisms and the use of appropriate methodologies—leading-edge or otherwise—comparative physiologists will continue to make vital contributions to mechanistic and evolutionary physiology. The esthetic lure of diverse biological systems will continue to attract to the field individuals who possess a desire to explore nature's variation. Through exploitation of the logic of comparative analysis, study of this natural variation will continue to yield insights about the most basic workings of organisms and the pathways by which their component systems have arisen and have been modified to permit life in the incredible diversity of environments in which it is found.

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