THE FUTURE OF PHYSIOLOGY: 2020 AND BEYOND

EDITED BY: George E. Billman and Geoffrey A. Head
PUBLISHED IN: Frontiers in Physiology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88971-166-6
DOI 10.3389/978-2-88971-166-6

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THE FUTURE OF PHYSIOLOGY: 2020 AND BEYOND

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This Research Topic eBook includes articles from Volume I and II of The Future of Physiology: 2020 and Beyond series:
Research Topic "The Future of Physiology: 2020 and Beyond, Volume I"
Research Topic "The Future of Physiology: 2020 and Beyond, Volume II"

INTRODUCTION

The term Physiology was introduced in the 16th century by Jean Francois Fernel to describe the study of the normal function of the body as opposed to pathology, the study of disease. Over the ensuing centuries, the concept of physiology has evolved and a central tenet that unites all the various sub-disciplines of physiology has emerged: the quest to understand how the various components of an organism from the sub-cellular and cellular domain to tissue and organ levels work together to maintain a steady state in the face of constantly changing and often hostile environmental conditions. It is only by understanding normal bodily function that the disruptions that leads to disease can be identified and corrected to restore the healthy state.

During the summer of 2009, I was invited by Dr. Henry Markram, one of the founders of the "Frontiers In" series of academic journals, to serve as the Field Chief Editor and to launch a new Open-access physiology journal that would provide a forum for the free exchange of ideas and would also meet the challenge of integrating function from molecules to the intact organism. In considering the position, I needed to answer two questions: 1) What exactly is Open-access publishing?; and 2) What could Frontiers in Physiology add to the already crowded group of physiology related journals?

As a reminder, the traditional model of academic publishing "is a process by which academic scholars provide material, reviewing, and editing expertise for publication, free of charge, then pay to publish their work" and, to add insult to injury, they and their colleagues must pay the publisher a fee (either directly or via an institutional subscription) to read their published work [slightly modified from the "The Devil's Dictionary of Publishing" Physiology News (the quarterly newsletter of the Physiological Society) Spring 2019: Issue 114, page 8]. In the traditional model, the publisher, not the authors, owns the copyright such that the author must seek permission and may even be required to pay a fee to re-use their own material (such as figures) in other scholarly articles (reviews, book chapters, etc.). In contrast, individuals are never charged a fee to read articles published in open-access journals. Thus, scholars and interested laymen can freely access research results (that their tax dollars paid for!) even if their home institution does not have the resources to pay the often exorbitant subscription fees. Frontiers takes the open-access model one step further by allowing authors (rather than the publisher) to retain ownership (i.e., the copyright) of their intellectual property.

Having satisfied the first question, I then considered whether a new physiology journal was necessary. At that point in time there were no open-access physiology journals, and further, many aspects of physiology were not covered in the existing journals. Frontiers afforded the unique opportunity to provide a home for more specialized sections under the general field journal, Frontiers in Physiology, with each section having an independent editor and editorial board. I therefore agreed to assume the duties of Field Chief Editor in November 2009. Frontiers in Physiology was launched in early 2010 and the first articles were published in April 2010. Since these initial publications, we have published over 10,000 articles and have become the most cited physiology journal. Clearly we must be fulfilling a critical need.

Now that it has been over a decade since Frontiers in Physiology was launched, it is time to reflect upon what has been accomplished in the last decade and what questions and issues remain to be addressed. Therefore, it is the goal of this book to evaluate the progress made during the past decade and to look forward to the next. In particular, the major issues and expected developments in many of the physiology sub-disciplines will be explored in order to inspire and to inform readers and researchers in the field of physiology for the year 2020 and beyond. A brief summary of each chapter follows:

In chapter 1, Billman provides a historical overview of the evolution of the concept of homeostasis. Homeostasis has become the central unifying concept of physiology and is defined as a self-regulating process by which a living organism can maintain internal stability while adjusting to changing external conditions. He emphasizes that homeostasis is not static and unvarying but, rather, it is a dynamic process that can

change internal conditions as required to survive external challenges and can be said to be the very basis of life. He further discusses how the concept of homeostasis has important implications with regards to how best to understand physiology in intact organisms: the need for more holistic approaches to integrate and to translate this deluge of information obtained in vitro into a coherent understanding of function in vivo. In chapter 2, Aldana and Robeva explore the emerging concept of the holobiont: the idea that every individual is a complex ecosystem consisting of the host organism and its microbiota. They stress the need for multidisciplinary approaches both to investigate the symbiotic interactions between microbes and multicellular organisms and to understand how disruptions in this relationship contributes to disease. This concept is amplified in chapter 3 in which Pandol addresses the future of gastrointestinal physiology ,emphasizing advances that have been made by understanding the role that the gut microbiome plays in both health and in disease. Professor Head, in chapter 4, describes areas in the field of integrative physiology that remain to be examined, as well as the potential for genetic techniques to reveal physiological processes. The significant challenges of developmental physiology are enumerated by Burggren in chapter 5. In particular, he analyzes the effects of climate change (environmentally induced epigenetic modification) on phenotype expression. In chapter 6, Ivell and Annad-Ivell highlight the major differences between the reproductive system and other organ systems. They conclude that the current focus on molecular detail is impeding our understanding of the processes responsible for the function of the reproductive organs, echoing and amplifying the concepts raised in chapter 1. In chapter 7, Costa describes the role of both circadian and non-circadian biological "clocks" in health and disease, thereby providing additional examples of integrated physiological regulation. Coronel, in chapter 8, provides a brief history of the development of cardiac electrophysiology and then describes areas that require further investigation and includes tables that list specific questions that remain to be answered. In a similar manner, Reiser and Janssen (chapter 9) summarize some of the advancements made in striated muscle physiology during the last decade and then discuss likely trends for future research; to name a few examples, the contribution of gender differences in striated muscle function, the mechanisms responsible of age-related declines in muscle mass, and role of exosome-released extracellular vesicles in pathophysiology. Meininger and Hill describe the recent advances in vascular physiology (chapter 10) and highlight approaches that should facilitate our understanding of the vascular processes that maintain health (our old friend homeostasis) and how disruptions in these regulatory mechanisms lead to disease. They also stress the need for investigators to exercise ethical vigilance when they select journals to publish in and meetings to attend. They note that the proliferation of profit driven journals of dubious quality threatens the integrity of not only physiology but science in general. The pathophysiological consequences of diabetes mellitus are discussed in chapters 11 and 12. In chapter 11, Ecelbarger addresses the problem of diabetic nephropathy and indicates several areas that require additional research. In chapter 12, Sharma evaluates the role of oxidative damage in diabetic retinopathy, and then proposes that the interleukin-6-trans-signaling pathway is a promising therapeutic target for the prevention of blindness in diabetic pateints. Bernardi, in chapter 13, after briefly reviewing the considerable progress that has been achieved in understanding mitochondrial function, lists the many questions that remain to be answered. In particular, he notes several areas for future investigation including (but not limited to) a more complete understanding of inner membrane permeability changes, the physiology of various cation channels, and the role of mitochondrial DNA in disease. In chapter 14, using Douglas Adam's

"The Hitchhikers Guide to the Universe" as a model, Bogdanova and Kaestner address the question why a young person should study red blood cell physiology and provide advice for early career scientists as they establish independent laboratories. They the, describe a few areas that merit further attention, not only related to red blood cell function, but also to understanding the basis for blood related disease, and the ways to increase blood supplies that are not dependent on blood donors. Finally, the last two chapters specifically focus on non-mammalian physiology. In chapter 15, Scanes asks the question, are birds simply feathered mammals, and then reviews several of the significant differences between birds and mammals, placing particular emphasis on differences in gastrointestinal, immune, and female reproductive systems. In the final chapter (chapter 16) Anton and co-workers stress that since some 95% of living animals species are invertebrates, invertebrate physiology can provide insights into the basic principles of animal physiology as well as how bodily function adapts to environmental changes. The future of Physiology is bright; there are many important and interesting unanswered questions that will require further investigation. All that is lacking is sufficient funding and a cadre of young scientists trained to integrate function from molecules to the intact organism.

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Citation: Billman, G. E., Head, G. A., eds. (2021). The Future of Physiology: 2021 and Beyond. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-166-6

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frontiers in Physiology

REVIEW
published: 10 March 2020
doi: 10.3389/fphys.2020.00200

Homeostasis: The Underappreciated and Far Too Often Ignored Central Organizing Principle of Physiology

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The grand challenge to physiology, as was first described in an essay published in the inaugural issue of Frontiers in Physiology in 2010, remains to integrate function from molecules to intact organisms. In order to make sense of the vast volume of information derived from, and increasingly dependent upon, reductionist approaches, a greater emphasis must be placed on the traditional integrated and more holistic approaches developed by the scientists who gave birth to physiology as an intellectual discipline. Our understanding of physiological regulation has evolved over time from the Greek idea of body humors, through Claude Bernard's "milieu intérieur," to Walter Cannon's formulation of the concept of "homeostasis" and the application of control theory (feedback and feedforward regulation) to explain how a constant internal environment is achieved. Homeostasis has become the central unifying concept of physiology and is defined as a self-regulating process by which an organism can maintain internal stability while adjusting to changing external conditions. Homeostasis is not static and unvarying; it is a dynamic process that can change internal conditions as required to survive external challenges. It is also important to note that homeostatic regulation is not merely the product of a single negative feedback cycle but reflects the complex interaction of multiple feedback systems that can be modified by higher control centers. This hierarchical control and feedback redundancy results in a finer level of control and a greater flexibility that enables the organism to adapt to changing environmental conditions. The health and vitality of the organism can be said to be the end result of homeostatic regulation. An understanding of normal physiology is not possible without an appreciation of this concept. Conversely, it follows that disruption of homeostatic mechanisms is what leads to disease, and effective therapy must be directed toward re-establishing these homeostatic conditions. Therefore, it is the purpose of this essay to describe the evolution of our understanding of homeostasis and the role of physiological regulation and dysregulation in health and disease.

Keywords: physiology, homeostasis, internal milieu, Claude Bernard, Walter Cannon, control theory, feedback regulation—negative and positive, cybernetics

OPEN ACCESS

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Specialty section:
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology

Received: *31 October 2019*
Accepted: *21 February 2020*
Published: *10 March 2020*

Citation:
Billman GE (2020) Homeostasis: The Underappreciated and Far Too Often Ignored Central Organizing Principle of Physiology. Front. Physiol. 11:200. doi: 10.3389/fphys.2020.00200

INTRODUCTION

In November 2009, I agreed to launch a new open-access physiology journal to be called Frontiers in Physiology and the articles were published in April 2010. One of my duties as Field Chief Editor was to write a brief "Grand Challenge" article in which I discussed what I perceived to be the biggest challenges facing physiology as a discipline. As it has been 10 years since the publication of this first essay, it is an opportune time to re-visit and update this grand challenge article.

THE GRAND CHALLENGE IN PHYSIOLOGY

In my 2010 essay, I stated that the grand challenge of physiology was "to integrate function from molecules to man" (Billman, 2010). In other words, to make sense of the vast volume of information derived from, and increasingly dependent upon, reductionist approaches. This, in my opinion, remains the most serious unmet challenge facing physiology today. A greater emphasis must be placed on the traditional integrated and more holistic approaches developed by the scientists who gave birth to physiology as an intellectual discipline. In other words, it time for physiologists to return our roots. It is no more possible to appreciate the beauty of de Vinci's "Mona Lisa" or Van Gogh's "The Starry Night" by removing and analyzing each individual dab of paint than we can understand how the various organ systems work together to maintain health by examining single genes or molecules. Just as when viewing a painting, the body can only be fully appreciated in its entirety. This essay will focus on the concept of homeostasis as the central organizing principle upon which the discipline of physiology is built, the very concept we need to return to in order to integrate function from molecule to the intact organism. Portions of the following sections were previously published in a slightly different form (Billman, 2013) and are reprinted with permission of the publisher.

HOMEOSTASIS: A DEFINITION

Homeostasis, as currently defined, is a self-regulating process by which biological systems maintain stability while adjusting to changing external conditions. This concept explains how an organism can maintain more or less constant internal conditions that allow it to adapt and to survive in the face of a changing and often hostile external environment. Our awareness of homeostasis has slowly emerged over the centuries and has become the central organizing tenet of physiology. If one does not understand this self-regulating process, then it is not possible to comprehend fully the function of the body in health and in disease. The disruption of homeostatic mechanisms is what leads to disease, and effective therapy must be directed toward re-establishing these homeostatic conditions, working with rather than against nature. In the following sections, the evolution of our understanding of homeostasis will be described and the role of physiological regulation and dysregulation in health and disease will be evaluated.

HOMEOSTASIS: A HISTORICAL PERSPECTIVE

> "*True stability results when presumed order and presumed disorder are in balance. A truly stable system expects the unexpected, is prepared to be disrupted, waits to be transformed.*"
>
> Tom Robbins (American Novelist, b. 1936)[1]

The concept that bodily regulation is required for health can be traced back to the ancient Greeks. The Greek physician/philosopher Alcmaeon of Croton (fl. 500 BC) proposed what can be called a "balance of opposites" to explain health and disease. He used a political analogy to define health and disease stating that: "*Health is the equality of rights of the functions, wet-dry, cold-hot, bitter-sweet and the rest; but single rule of either pair is deleterious.*" (Freeman, 1948). Thus, inequality of power leads to tyranny in a political system and disease in the body. This concept was expanded by Hippocrates of Kos (ca. 460–ca. 377 BC) who proposed that health was the product of the balance and mixture of four body fluids or humors: blood, phlegm, yellow bile, and black bile. He wrote that:

> "*Health is primarily that state in which these constituent substances are in correct proportion to each other, both in strength and quantity and are well mixed. Pain occurs when one of these substances presents either a deficiency or excess, or is separated in the body and not mixed with the others.*" (Chadwick and Mann, 1950)

Thus, medicine became a process "*of subtraction and addition: subtraction of what is in excess, addition of what is wanting.*" (Jones, 1923). Hippocrates further recognized the role of nature's helping hand in the healing process (*vis medicatrix naturae*), the ability of the body to heal itself (Hall, 1975). It was the role of the physician to clear the path so that nature could take its course. This concept became the basis for medicine in the ensuing centuries up to the dawn of the modern era.

Implicit in this concept of the "healing power of nature" is the assumption that the subunits of the body act in a cooperative manner to restore health when the normal state of the organism has been disturbed. Physiology, as a discipline dedicated to understanding how the parts of the body work together to maintain health, has its origins in the 16th century. The term physiology was first introduced by Jean Francois Fernel (ca. 1497–1558, **Figure 1**) in 1542 [*De Naturali Parte Medicinae* (on the natural part of medicine)] as the study of the function of the healthy body as distinguished from pathology, the study of disease (Hall, 1975). William Harvey (1578–1657) was the first individual to use carefully designed human and animal experiments to establish the function of a major bodily organ system with his description of the circulation of the blood. This application of physiology is illustrated in the following brief quotation from his seminal publication "*Exercitatio Anatomica De Motu Cordis et De Circulatione Sanguinis in Animalibus*" 1628 (Anatomical exercises on the motion of the heart and the circulation of blood in living creatures, first English translation 1653):

[1] http://www.brainyquote.com/quotes/quotes/t/tomrobbins404093.html

FIGURE 1 | Portrait of Jean Fernel (ca. 1497–1558). He is the individual who coined the term physiology. Source: National Library of Medicine (the history of medicine public domain image files).

FIGURE 2 | Photograph of Claude Bernard (1813–1878). He developed the concept of "*a fixité du milieu intérieur*," that is, organisms maintain a stable internal environment despite changing external conditions. Source: National Library of Medicine (the history of medicine public domain image files).

> "It has been shown by reason and experiment that blood by the beat of the ventricles flows through the lungs and is pumped to the whole body . . . the blood in the animal body moves around in a circle continuously, and . . . the action or function of the heart is to accomplish this pumping. This is the only reason for the motion and beat of the heart." (Harvey, 1628/1653)

Over the ensuing centuries, the concept of physiology has evolved, and a central tenet has emerged that unites the various sub-disciplines of physiology: the quest to understand how the various components of the organism work together to maintain a healthy state. It is only by understanding normal bodily function that the disruptions that lead to disease can be determined and ultimately corrected so as to restore the healthy state.

As we have seen, a rudimentary understanding of the regulation and control of bodily function can be traced back to 6th century BC Greece. Despite sporadic progress over the centuries (Adolph, 1961), it was not until the 19th century that systematic physiological investigation produced major advancements on this concept. Our modern understanding of physiological regulation rests firmly on the shoulders of two giants in the field: Claude Bernard (**Figure 2**) and Walter Cannon (**Figure 3**) who described regulations in terms of the constancy of the internal environment and homeostasis, respectively.

The French Physiologist, Claude Bernard (1813–1878), who is often referred to as the founder of modern experimental physiology, was perhaps the first to appreciate fully that living systems possess an internal stability that buffers and protects the organism against a constantly changing external environment (Cooper, 2008). He recognized that the body possesses mechanisms that operate in a coordinated fashion to maintain a relatively constant temperature and blood glucose concentration and this internal stability was vital for the health of the organism. He concluded that: "*La fixité du milieu intérieur est la condition de la vie libre, independante*" (Bernard, 1865) [The fixity (i.e., constancy or stability) of the internal environment is the condition for the free, independent life]. What is often

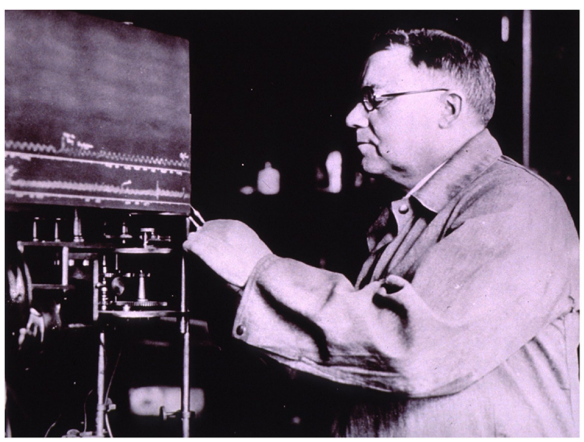

FIGURE 3 | Photograph of Walter B. Cannon (1871–1945). He built upon the work of Claude Bernard and coined the word homeostasis to describe a self-regulating process by which biological systems maintain stability while adjusting to changing conditions. Source: National Library of Medicine (the history of medicine public domain image files).

overlooked and needs to be stressed is that in this statement Bernard was proposing a new and radical hypothesis: the stability of the "*milieu intérieur*" was the antecedent to (i.e., required for) and not the consequence (outcome) of a free and independent life (Turner, 2017).

Although Bernard was highly honored and was the most famous French scientist during his lifetime, his hypothesis that the stability of the internal environment was independent of the external conditions, first articulated in 1854, was largely ignored for the next 50 years. Gross (2009) has proposed three reasons to explain the delay between the publication of Bernard's ideas and their acceptance: (1) Pasteur's exciting discoveries in bacteriology that had immediate application in the prevention and treatment of disease came to dominate biological investigations; (2) the gap between evolutionary thought and general physiology—it took time to appreciate that natural selection provided the means by which regulatory control could evolve; and (3) the technology necessary to measure the internal environment was not yet available.

However, by the late 19th century and early 20th century several investigators embraced Bernard's ideas, both as a central explanatory concept and as a program for research in physiology. Among those influenced by Bernard were such physiological luminaries as William M. Bayliss, Ernest H. Starling, Joseph Barcroft, J. S. Haldane, and C. S. Sherrington in England, and L. J. Henderson and Walter B. Cannon in America (Adolph, 1961; Cooper, 2008; Gross, 2009). Starling, in fact, coined the phrase "the wisdom of the body" to describe the maintenance of a constant internal environment (Cooper, 2008). Walter Cannon later popularized this phrase when he used it as the title for his book in which he introduced the concept of homeostasis. In 1900, Charles R. Richet (1850–1935), a student of Bernard who later won the Nobel Prize in Physiology and Medicine, stressed the dynamic stability of the internal environment. The following quote, we shall see, presaged the definition supplied by Walter Cannon.

> "*The living system is stable. . .it must be in order not to be destroyed, dissolved or disintegrated by colossal forces, often adverse, which surround it. By an apparent contradiction, it maintains its stability only if it is excitable and capable of modifying itself according to external stimuli and adjusting its response to the stimulation. In a sense, it is stable because it is modifiable – the slight instability is the necessary condition for the true stability of the organism.*" (Richet, 1900)

This concept of a constant internal environment (*milieu intérieur*) was expanded by the American Physiologist, Walter Cannon (1871–1945) (Cooper, 2008). He coined the term homeostasis from the Greek words Ὅμοιος (hómoios) "similar" and στάσις (stásis) "standing still" (together to mean staying similar and *not* staying the same) to describe the self-regulating processes by which a biological system maintains stability while adjusting to changing environmental conditions. Homeostasis is often mistakenly taken to mean unchanging or stagnant. However, Cannon purposely selected the Greek word for similar, "hómoios," rather than the word for same, "homo," to express the idea that internal conditions could vary; that is, they are similar but not identical (stability but within range of values that allows the organism the freedom to adapt). Homeostasis, then, is the tendency of a system to maintain an internal stability as the result of the coordinated response of its parts to any situation or stimulus that disturbs normal conditions or function. Thus, the term homeostasis attempts to convey two ideas: (1) an internal stability within a range of values and (2) the coordinated dynamic response that maintains this internal stability (self-regulatory goal-seeking behavior). As he explained in the following quote from his highly influential monograph, "The Wisdom of the Body," published in 1932:

> "*The coordinated physiological processes which maintain most of the steady states in the organisms are so complex and peculiar to living beings – involving, as they may, the brain and nerves, the heart, lung, kidneys and spleen, all working cooperatively – that I have suggested a special designation for these states, homeostasis. The word does not imply, something set and immobile, a stagnation. It means a condition – a condition which may vary, but is relatively constant.*" (Cannon, 1963)

As emphasized by Cannon, homeostasis is not static; it is, rather, a dynamic self-adjusting system that maintains viability in the face of changing environmental demands. Echoing Bernard, homeostasis is a unique property of living organisms and, may be responsible for life itself. More recently, Turner (2017) described homeostasis as a dynamic disequilibrium – dynamic, as a stable internal environment requires continuous monitoring and adjustments (once again, a self-regulatory process) in order to maintain a balance between opposing forces (what he calls disequilibrium) so that a free and independent life is possible. He went further and stated that "*properly understood, homeostasis is life's fundamental property, what distinguishes it from non-life. In short, homeostasis is life.*" (Turner, 2017).

The final piece of the homeostasis puzzle was supplied by the application of control theory from systems engineering to explain self-regulation in biological systems. The "constancy" of internal physiochemical conditions is then largely maintained by the often complex interaction of multiple negative (and positive) feedback systems. The interaction of these regulatory mechanisms not only increases the stability of the system but provides redundancy (back-up) such that failure of one component does not necessarily lead to catastrophe. Thus, from its inception physiological investigations have been directed toward understanding the organism (be it microbe, plant, animal, or human) as a *single functional entity*.

FEEDBACK REGULATION: THE PROCESS THAT UNDERLIES HOMEOSTASIS

> "*Nam deteriores omnes sumus licentiate.*" (*We all degenerate in the absence of control*)
>
> Terence (Heauton Timorumenos, line 483)

As we have seen, a critical feature of homeostasis is that an organism's internal environment is held within a narrow

range of values via a self-adjusting (a goal-seeking) system. Both feedback and feedforward are the mechanisms by which homeostasis is obtained. I shall begin this section with a discussion of the contribution of feedback to homeostatic regulation and then briefly discuss feedforward (also known as central command) mechanisms.

A feedback system is a closed loop structure in which the results of past actions (changes in the internal environment) of the system are fed into the system (via information, feedback) to control future action; the system affects its own behavior (modified from Forrester, 1976). There are two types of feedback systems: negative feedback that seeks a goal and responds as a consequence of failure to meet this goal (maintains a stable *range* of values) and positive feedback that produces growth processes wherein the actions build on the results that then generate still greater action (a growth cycle). These feedback systems are themselves subject to higher levels of control; that is, the operational range of the regulated variables can be adjusted to support the behavioral response to environmental stimuli. Homeostasis is the result of the complex interaction and competition between multiple negative and positive feedback systems and provides the basis for physiological regulation.

Once again we can trace the origin of self-regulatory systems to the ancient Greeks.

The first documented device that employed the principle of self-regulation was a water clock (clepsydra) invented by Ktesibios (or Ctesibius, Greek Κτησίβις) of Alexandria (fl. 285-222 BC) (Landels, 2000). A water clock depends upon a steady flow of water to measure an unvarying flow of time. If the water level is not relatively constant, the water outflow will vary depending on the height of the water column supplying the clock (faster with a full container and slower as the water level in the container falls). The water clock designed by Ktesibios used a float valve (similar to that used in the modern flush toilet) to maintain a constant water level in the clock water reservoir. As water levels fall, the float also falls thereby opening a valve that allows water to flow into the clock reservoir and to replenish the water level. Then, as the water returns to the desired level, the float rises and closes the valve. Thus, the clock water reservoir could be regulated such that there is no net gain or loss in the water level and thereby it maintains a constant water outflow rate from which an accurate estimate of time can be obtained. The accuracy of this type of water clock was not supplanted until the 17th century when a pendulum was employed to regulate the clock mechanism.

A number of other self-regulatory devices were invented in the ancient and medieval periods but it was not until the late 18th century, with the invention of the steam engine that the study of devices that incorporated "corrective feedback" for regulation became a subject for systematic investigation. A major limitation of early steam engines was that their speed was affected by both the steam pressure generated by the boiler and work load placed upon the engine. James Watt (1736–1819) vastly improved the efficiency and safety of the steam engine by the development of a centrifugal feedback valve that controlled the speed of the engine (Rosen, 2010). This "governor" (**Figure 4**) employed a pair of metal balls spinning on each side of a rotating vertical shaft aligned in such a manner that as the engine speed increased so also did the spinning rate of metal balls (called flyweights) and, as a consequence of increased centrifugal force, the balls would spread apart. This, in turn, opened a valve to decrease the flow of steam into the engine and a slower speed was restored. Conversely, as the engine speed decreased, so also would the rotation of the flyweights, thereby decreasing the outward centrifugal force. The flyweights would drop (pulled down by gravity) closer together, closing the steam valve so more steam could enter into the engine and increase its speed. As with the water clock and its water reservoir level, a constant engine speed could be maintained despite fluctuating steam pressure and changing work load without the constant supervision of a human monitor.

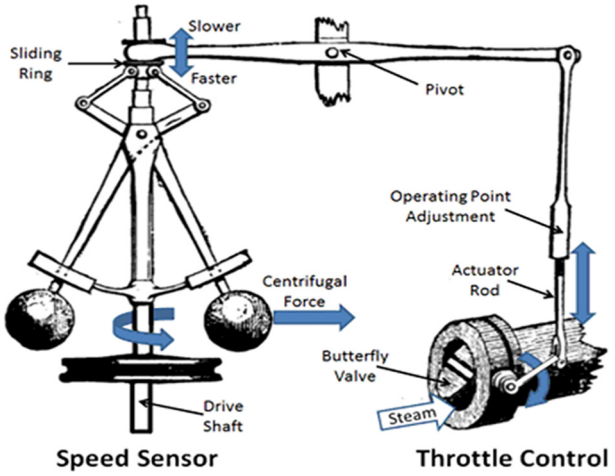

FIGURE 4 | Schematic representation of James Watt's steam engineer flyweight governor. See text for details. Source: public domain, as modified from, https:www.mpoweruk.com/figs/watt_flyball_governor.htm.

Later in the 19th century, James Clerk Maxwell (1831–1879) published a mathematical analysis of Watt's governor that established the principles for understanding self-regulating devices and became the foundation upon which control theory is built (Maxwell, 1868). In 1927, Harold S. Black (1898–1983) applied feedback regulation to electrical circuits to amplify transatlantic telephone signals (Black, 1934). His negative feedback amplifier (patented in 1937) can be considered to be one of the most important developments in the field of electronics. Further advances in systems control theory were achieved during World War II with the development of servo-control (negative feedback) mechanisms for anti-aircraft weapons.

In 1943, two influential papers were published that established that the mathematical principles of control theory, as first described by Maxwell, could be applied to explain behavior in living organisms. Arturo Rosenblueth, Norbert Wiener, and Julian Bigelow's paper entitled "Behavior, Purpose and Teleology" (Rosenblueth et al., 1943) and Warren McCulloch and Walter Pitts', "A Logical Calculus of the Ideas Immanent in the Nervous Activity" (McCulloch and Pitts, 1943) were the first to establish a link between the self-regulating nature of physiological

processes in living animals and negative-feedback systems designed by engineers. Interestingly, Rosenblueth worked closely with Cannon and undoubtedly was influenced by his ideas. A few years later, Wiener (1894–1964) introduced the term cybernetics [from kybernetes (κυβερνήτης), the Greek word for governor (as in steersman or pilot)] to describe the study of self-regulatory control and communication in the animals (Wiener, 1961). In his book Cybernetics, Wiener (1961) developed the first formal mathematical analysis of feedback control in biological systems, concepts that have subsequently been extensively applied in modeling physiological systems as, for example, by Arthur Guyton (1919–2003) and his many students with regard to cardiovascular regulation. Thus, the concept of feedback regulation in living organisms may be said to have co-evolved with the mathematical concepts of control theory in mechanical systems. Negative feedback regulation is a particularly important mechanism by which homeostasis is achieved, as will be described in the following paragraphs.

The water clock and centrifugal steam governor described in the preceding paragraphs provide classic examples of negative feedback systems. As we have seen for the water clock, the opening and closing of the float/valve creates a cycle where information about the water level can be fed back into the system to effect changes to maintain the water level at some constant predetermined value. Thus, the float simultaneously affects the water levels and is affected by water level forming a circular causality or a cycle of causation. It is important to emphasize that this is an automatic self-regulatory system, meaning that it requires no external adjustment once the operating level around which the variable is regulated has been set.

A simplified general form of a closed loop feedback system is illustrated in **Figure 5**. The illustrated cycle consist of four main components, (1) the variable (or set of variables) that are to be controlled, (2) a sensor that monitors the variable of interest, (3) a comparator or central processing unit (mathematically, the transfer function—the input/output relationship) where the information provided by the sensor (afferent or sensory pathway) is fed back into the system. The information is compared with the "desired" state (set point or operating point) to detect any error (difference between the desired state and the prevailing state), and (4) effectors (efferent or motor pathways) that are activated to correct any error. Effector activity opposes and thereby buffers against changes in the variable. A solid line is used in this diagram to indicate a direct relationship (increase leads to increase, decrease leads to decrease) between the components, while a dashed line represents an inverse relationship (increase leads to a decrease and vice versa). Negative feedback regulation must contain an odd number of dashed lines in order to maintain the variable within a narrow range of the desired value.

A commonly used example of negative feedback is the regulation of room temperature by a thermostatically controlled heating and cooling system as displayed in **Figure 6**. Room temperature is the regulated variable, the sensor is a thermometer, the comparator is the thermostat—the device that compares the desired temperature (operating point) with the actual temperature (error detection), and the effector is the heating or cooling system. In this example, an increase in outside heat is detected by the sensor and the information is conveyed to the thermostat. The temperature information is compared to operating point and if there is sufficient difference between actual and desired temperature, the cooling system is activated and

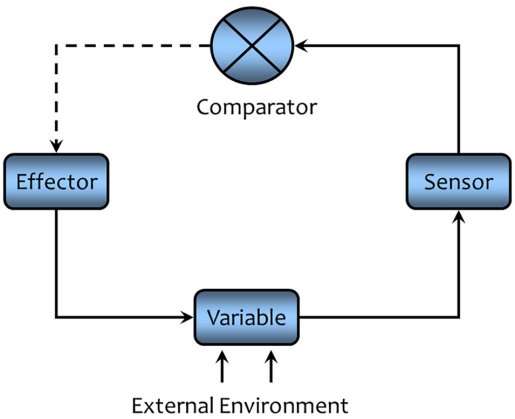

FIGURE 5 | A schematic representation of negative feedback regulation. A solid line indicates that the connected components are directly related (an increase in one component leads to increase the connected component, while a decrease will lead to decrease in the connected components). A dashed line indicates the connected components are inversely related (an increase in one component leads to a decrease in the connected component while a decrease will lead to an increase in the connected component). An odd number of dashed lines are a necessary condition for any negative feedback cycle of causation. Negative feedback acts to maintain the controlled variable within a narrow range of values (see text for a detailed description).

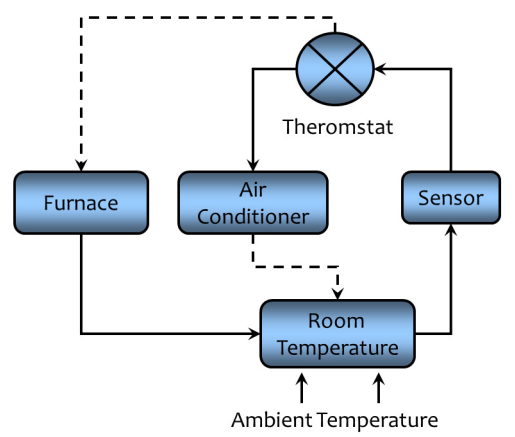

FIGURE 6 | A schematic representation of the regulation of room temperature to illustrate the concept of negative feedback regulation. A solid line indicates that the connected components are directly related (an increase in one component leads to an increase the connected components, while a decrease will lead to a decrease in the connected components). A dashed line indicates that the connected components are inversely related (an increase in one component leads to a decrease in the connected component while a decrease will lead to an increase in the connected component). Negative feedback acts to maintain the room temperature within a narrow range of values despite changes in ambient temperature (see text for a detailed description).

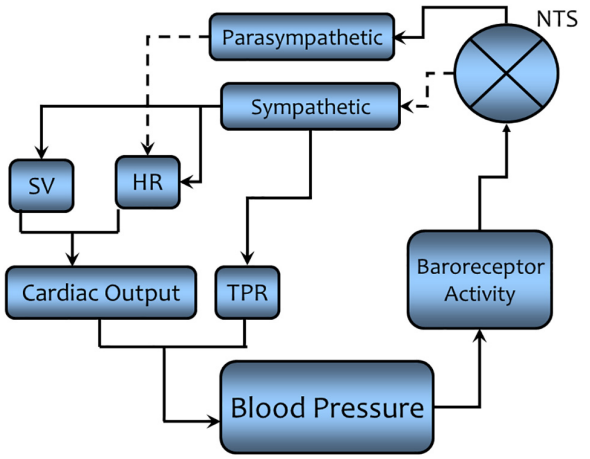

FIGURE 7 | A simplified schematic representation of the regulation of arterial blood pressure as a physiological example of negative feedback regulation. A solid line indicates that the connected components are directly related (an increase in one component leads to an increase the connected components, while a decrease will lead to a decrease in the connected components). A dashed line indicates the connected components are inversely related (an increase in one component leads to a decrease in the connected component while a decrease will lead to an increase in the connected component). Negative feedback regulation acts to maintain the arterial blood pressure within a narrow range of values (see text for a detailed description). NTS = nucleus tractus solitarius, the site where sensory information is processed and the efferent response is initiated. It acts as a "barostat" analogous to the "thermostat" in room temperature regulation. SV = stroke volume (the amount of blood ejected by the heart with each ventricular contraction), HR = heart rate, the number of beats (ventricular contractions) per minute, TPR = total peripheral resistance, the resistance to the forward movement of blood (inversely related to the blood vessel diameter).

the heating system is inactivated (reducing the error signal). The converse would happen if environmental temperature should fall, the cooling system would be turned off and the heating units activated. Thus, stable room temperatures can be maintained despite a wide range of fluctuating external conditions.

It must be emphasized that feedback regulation in biological systems (living organisms) is much more complex than the simple "clockwork" feedback systems described in the preceding paragraphs for mechanical systems. With this caveat firmly in mind, the concept of self-regulation in biological system can be illustrated by the regulation of blood pressure. As early as the mid-19th century, it became obvious that arterial blood pressure was maintained within a narrow range of values via the activation of neutrally mediated reflex adjustments (Adolph, 1961). However, it was not until to 1960s that the principles of negative feedback were applied to explain the homeostatic regulation of arterial blood pressure. A detailed description of intricacies of blood pressure regulation is beyond the scope of the present essay (for a recent review see Dampney, 2016). Nonetheless, a *simplified* feedback cycle, analogous to the one we used for room temperature, is seen in **Figure 7**.

Before we can discuss this figure, we first must mathematically define arterial pressure using Ohm's law expression (for a hydraulic rather than for an electrical circuit). Algebraically, blood pressure (BP – analogous to voltage, E, in an electrical circuit) is the product of the cardiac output (CO – analogous to current, I, in an electrical circuit) and systemic vascular resistance also known as total peripheral resistance (TPR – analogous to electrical resistance, R). Cardiac output is itself the product of the amount of blood ejected per beat [stroke volume (SV)] multiplied by the number of beats per minute [heart rate (HR)].

So that, BP = SV × HR × TPR. (E = I × R for an electrical circuit).

It is evident that changes in arterial blood pressure can be countered by corrective changes in either the output from the heart (SV and/or HR) or resistance to movement of blood through blood vessel (by adjusting vessel diameter, diameter is inversely related to TPR) or both. Returning to **Figure 7**, the sensors are receptors (baroreceptors) located in arterial blood vessels (aortic arch and carotid sinuses) that respond to changes in arterial pressure (increases in BP increase receptor activity). The comparator function is performed by a cluster of nerve cells within the medulla of brain [nucleus tractus solitarius (NTS)] where the signal is processed to affect the output of the effector system. It acts as a "barostat" a function analogous to the thermostat in the regulation of room temperature shown in **Figure 6**. The signal is processed at the NTS and then effects excitatory [rostral ventral lateral medulla (RVLM) via interneuron connections] and inhibitory [nucleus ambiguus (NA), monosynaptically] areas within the medulla to elicit the motor response (see **Figure 8** for more details). The motor output from the central nervous system to target organs is conducted by means of two sets of nerves to the heart: parasympathetic nerves (originating in the NA) that decrease HR and sympathetic nerves (originating in the intermediolateral column, IML of the spinal cord, regulated by neurons from the RVLM) that increase HR and SV. The sympathetic nerves also go to blood vessels, the activation of which decreases vessel diameter and thereby increases TPR. Thus, if BP should increase, the so-called baroreceptor reflex is activated. An increase in parasympathetic activity coupled with a decrease in sympathetic activity would reduce cardiac output (decreasing HR and SV) and decrease TPR. The opposite changes would occur if blood pressure should decrease. Thus, negative feedback regulation buffers against transitory changes and thereby helps maintain a stable blood pressure on a beat-by-beat basis throughout the day despite changing environmental or behavioral conditions.

Feedforward regulation is another mechanism by which homeostasis is modified and maintained as part of the behavioral response to environmental stimuli. During feedforward regulation, which is also often referred to as central command, a response is elicited without feedback about the status of the regulated variable; that is, disturbances are evaluated and adjustments are made *before* changes in the regulated variable have actually occurred. For example, returning to constant room temperature, feedforward regulation would entail activation of the furnace as soon a window or door is opened during a cold winter day before the thermostat detects a change in the ambient temperature, In a similar manner, blood pressure,

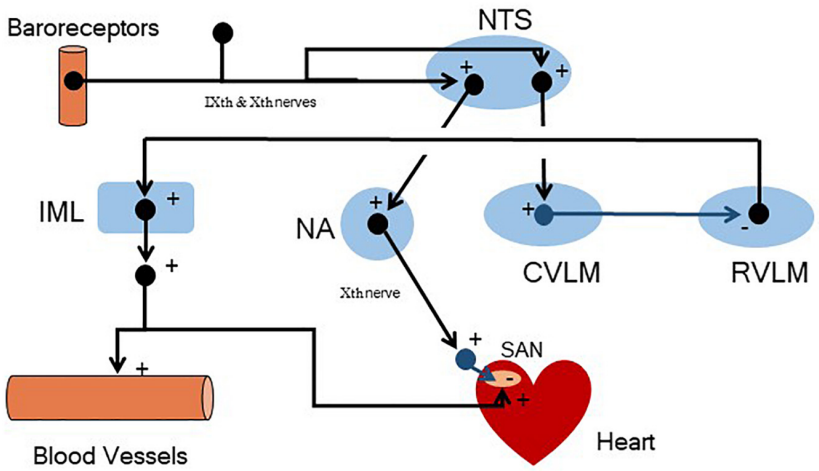

FIGURE 8 | A simplified schematic representation of the central neural structures involved in baroreceptor reflex regulation of arterial blood pressure. Arterial pressure receptors located in the carotid sinuses and aortic arch (nerve firing increases as arterial pressure increases) convey afferent information via the glossopharyngeal (IXth) and vagus (Xth) nerves to the brain, respectively. This information is first processed by neurons located in the nucleus tractus solitarius (NTS). The NTS then alters parasympathetic and sympathetic efferent nerve activity. Specifically, the NTS alters the activity of neurons (monosynaptically) located in the nucleus ambiguus (NA, parasympathetic pre-ganglionic neurons) and neurons (polysynaptically, via interneuron connections) in the caudal ventrolateral medulla (CVLM). The CVLM, in turn, regulates the tonic sympathetic activity that originates in the rostral ventrolateral medulla [RVLM, that regulates sympathetic pre-ganglionic neurons located in the intermediolateral column (IML) of the spinal cord]. + = excitatory neurotransmitters (shown in black); – = inhibitory neurotransmitters (shown in blue); SAN = sino-atrial node. As an example, an increase in arterial blood pressure would increase baroreceptor nerve firing, increasing NTS neuron activity which, via interneurons, would trigger both an increase in the activity of the parasympathetic pre-ganglionic neurons located in the NA and decrease the firing of sympathetic pre-ganglionic neurons located in the IML (less directly via CVLM mediated inhibition of the tonic activity of the RVLM). The net result would be a decrease in heart rate (? cardiac parasympathetic and↓ cardiac sympathetic nerve activity), stroke volume (↓ cardiac sympathetic nerve activity), and arteriolar vasoconstriction (↓ total peripheral resistance, ↓ cardiac sympathetic nerve activity). Reductions in arterial blood pressure would provoke changes in the opposite direction. Note that the sign changes at the heart (parasympathetic effects on the SAN) and within the medulla (CVLM mediated inhibition of the RVLM). This "sign change" is necessary for negative feedback regulation.

cardiac output, and skeletal muscle blood increase in anticipation of fighting or fleeing a potential danger (the defense reaction) or when an athlete envisions running the race before the starter's pistol has been fired (see below). It should be emphasized that feedforward regulation, while acting independently of changes in the regulated variable, does require information about the nature and extent of the potential disturbance. For room temperature, the status of the windows and doors (whether they are open or not) must be monitored (sensors placed on these openings). Otherwise, a response would not be elicited until room temperature had deviated sufficiently from the set point to be detected by the thermostat (and thereby activate the previously described negative feedback response). In living organisms, learning and experience provide the information necessary for feedforward control. A cat soon learns the difference between a mouse (food) and the neighbor's dog (a dangerous and barking nuisance) and will react accordingly (making the appropriate behavioral and physiological adjustments for appetitive or aversive stimuli).

The simple negative feedback schema described in the preceding paragraph cannot adequately convey the complexity of the homeostatic process that allows an organism to function and adapt to changing environmental conditions (Carpenter, 2004). For example, the operating point (or more accurately the operating range) of the negative feedback regulation can be adjusted or even overridden by higher levels of control (Goodman, 1980). These adjustments of the automatic (e.g., feedback) regulation allow the organism to adapt and to respond appropriately to changing external conditions. This hierarchical control is a multi-level, multi-goal seeking system as shown in **Figure 9** (modified from Goodman, 1980). In this schematic diagram, the first level represents the physiochemical processes, the organ and tissue functions, the component parts upon which homeostasis acts. The second level is autonomous (self) regulation, homeostasis (e.g., baroreceptor reflex). Here changes in a given variable are sensed and adjustments of the first level processes are initiated without input from higher levels of control. The third level is found in the central command and control centers (central nervous system) that process the information transmitted from the second level and integrates it with information from other sensory inputs to coordinate the physiological and behavioral response to changing environmental conditions. The higher centers can "intervene," making the adjustments as required to support the autonomic (i.e., autonomous and automatic) processes. This control can occur either at the conscious or unconscious level. An example of a conscious intervention would be the initiation of behaviors to cope with changing room temperature – adding or removing clothing, opening or closing windows seeking shade or sun, etc. – while an example of subconscious control would be the adjustments in blood pressure regulation during exercise (a shift in the operating point of the baroreceptor reflex so

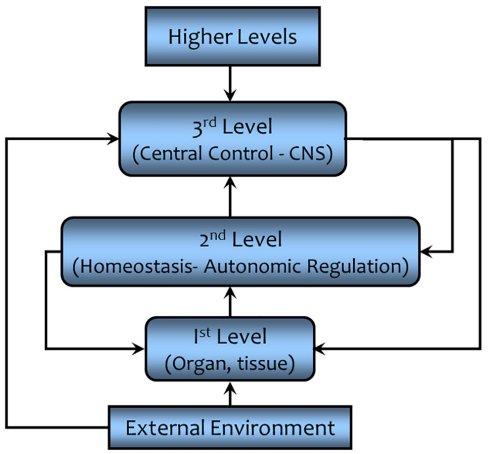

FIGURE 9 | A simplified schematic representation of the higher order control of homeostatic regulation. This hierarchical control results in a finer level of control and a greater flexibility that enables the organism to adapt to changing environmental conditions (see text for details). CNS = central nervous system.

that both HR and SV increase despite increases in BP as compared to resting conditions; Raven et al., 2006). Thus, the third level coordinates behavioral and physiological responses to the external environment in order to maintain comfort and to ensure survival. However, it must be emphasized that higher level control is not possible if the first level components do not function properly. Finally, one could also envision even higher levels of control, factors outside of the organism.

The "autopilot" in a modern jet airliner can be used to illustrate the levels of control (Wiener, 1961). Once the preferred heading, attitude, and airspeed have been set, the autopilot will maintain level flight within acceptable degrees of roll, pitch, and yaw, despite changes in wind speed or minor turbulence. However, take-off and landing (at least until "self-driving" technology has been perfected) require the direct intervention of the human pilot. Thus, the first level consists of the components of the airliner, the jet engines, and the airframe (fuselage, wings, flaps, rudder, etc.), the second level is the autopilot, and third level is the human pilot. In this example, a fourth level of control of the airplane is exerted by the air traffic controllers who provide directions to the pilot while an even higher level of control would reside in the Federal Aviation Administration (FAA) that sets the policy followed by the air traffic controllers.

The cardiorespiratory response to exercise provides a physiological example of this hierarchical control of homeostatic regulation. The first level consists of the tissues and organs that form the cardiovascular and respiratory system (heart, lung, and blood vessels, but also the kidneys and endocrine glands that regulate salt and water retention and thereby blood volume), the second level of control is the baroreceptor (direct effect) and cardiorenal reflexes (indirect via regulation of blood volume), the third level of regulation takes place within the medulla (NTS) of the central nervous system where the sensory information is processed and the efferent response initiated. The medullary structures are themselves regulated by higher centers (e.g., hypothalamus and motor centers) in the brain. In fact, the hypothalamus plays a major role in coordinating (matching) changes in the internal environment with the behavioral response to external challenges. As previously mentioned, HR and BP are simultaneously elevated during exercise demonstrating that baroreceptor reflex regulation has been altered. These adjustments are required in order to increase oxygen delivery so that it can match the increased metabolic demand of the exercising muscles. Raven et al. (2006) have demonstrated that these adjustments result from shifting the baroreceptor reflex to a higher operating point (i.e., altering the range of homeostatic regulation) rather than from an inhibition of this reflex. Both feedback (sensory information for the exercise muscle, the so-called exercise pressor reflex) and feedforward (central command: for example, anticipation of the onset of exercise, such as visualizing the race before it is run, will increase HR, BP, and skeletal muscle blood flow) contribute to these reflex adjustments. Finally, higher levels of control include the starter who determines when the race will begin, the event organizers who determine what races are run, and the sports regulatory agencies (Olympic committee, FIFA, NCAA, etc.) that set the rules that govern the event.

Homeostatic control of the internal environment, therefore, involves much more than simple negative feedback regulation (Carpenter, 2004). The hierarchical levels of command and control allow the organism to adjust its internal conditions to respond, to adapt, and to meet the challenges placed upon it by a changing and often hostile environment. Adaptation can, in fact, be viewed as an emergent property of homeostasis and may be responsible for the life's unique nature (Turner, 2017).

HOMEOSTASIS: IMPLICATION FOR REDUCTIONISM

"*. . .All the kings' horses and all the kings' men*
Could not put Humpty Dumpty together again"
Traditional English Nursery Rhythm
(earliest published version 1803)
(Opie and Opie, 1997)

The concept of homeostasis has important implications with regard to how best to understand physiology in intact organisms. In recent years, reductionist (attempts to explain the nature of complex phenomena by reducing them to a set of ever smaller and simpler components; the view that the whole is merely the sum of its parts), rather than holistic approaches have become dominant, not only in physiology, but in science in general. The earliest glimmerings of reductionist thought can be found in the surviving fragmentary writings of Thales and other pre-Socratic Greek philosophers who speculated that all matter was composed of various combinations of four key elements: earth, air, fire, and water (the four humors of the body correspond to these elements) (Hall, 1975). The pinnacle of Greek reductionism is found in the work of Leucippus and his student Democritus who proposed that all things consist of an infinitely large number of indivisibly small particles that they called atoms (Hall, 1975). The modern application of reductionism in science can be traced

to Francis Bacon (1561–1620) and Rene Descartes (1596–1650). Bacon incorporated reductionism as a central component, along with inductive reasoning, in his new empirical method (*Novum Organum 1620, as* opposed to Aristotle's *Organon* a treatise on logic and syllogism, i.e., deductive reasoning) (Bacon, 1620) for the attainment of knowledge in natural philosophy, what has subsequently become known as the scientific method. Descartes likewise embraced reductionism as the pathway to knowledge, albeit with an emphasis on deduction (rationalism) rather than induction (empiricism) as advocated by Bacon. In his "Discourse on the Method of Rightly Conducting One's Reason and Seeking Truth in Science," Descartes (1637) introduced two concepts that would have profound impact on biological investigations. In this, his most influential treatise, he described four precepts to arrive at knowledge. The second and third precepts, in particular, exemplify the reductionist's approach as follows:

> "*The second to divide each of the difficulties under examination into as many parts as possible and as might be necessary for its adequate solution*"
>
> "*The third to conduct my thoughts in such order that, beginning with those objects that are simplest and most readily understood, I ascend little by little, and as it were, step by step, to the knowledge of the more complex.*" (Descartes, 1637)

His second and more far reaching conclusion was that the body was merely a machine. Thus, it was assumed that by applying Cartesian reductionism, one could deduce the complex physiology of the intact organism by understanding the presumably simpler functions of the individual organs and their constituent parts (from the molecular level to subcellular organelles to cells to tissue to organ and finally back to the intact organism).

There can be no denying the power of this approach. In only a few decades after DNA was identified as the molecule of inheritance, its sequence of the some 3 billion base pairs has been mapped for humans and other species, the genetic "code" for protein synthesis has been broken, and between 20,000 and 25,000 human genes that regulate a multitude of proteins have been determined. Humpty Dumpty quite literally has been smashed into a billion pieces.

However, reductionism rests upon the unstated assumption that the parts somehow entail the whole, that complexity is merely the product of incomplete understanding. In other words, the assumption that once we have gathered enough information (big data) and have developed sufficient computing power (ultra-fast computers), we can put Humpty back together again. The salient question is then whether this assumption is correct? Although we have sequenced the genome for many species, we have little understanding of the process by which the genome becomes an organism. We now know, in intricate detail, the basis for neuronal action potentials and synaptic transmission but do not understand how these electrical and chemical events give rise to consciousness. Complexity may not be the illusion it once naïvely was thought to be. As elegantly described by Claude Bernard more than 150 years ago:

> "*Physiologist and physicians must never forget that a living being is an organism with its own individuality. Since physicists and chemists cannot take their stand outside the universe, they study bodies and phenomena in themselves and separately, without necessarily having to connect them with nature as whole. But physiologists, finding themselves, on the contrary, outside the animal organism which they see as a whole, must take account of the harmony of the whole, even while trying to get inside, so as to understand the mechanism of its every part. The result is that physicists and chemists can reject all idea of the final causes for the facts that they observe; while physiologists are inclined to acknowledge a harmonious and pre-established unity in an organized body, all of whose partial actions are interdependent and mutually generative. We really must learn, then, that if we break up a living organism by isolating its different parts, it is only for the sake of ease in experimental analysis, and by no means in order to conceive them separately. Indeed, when we wish to ascribe to a physiological quality its value and true significance, we must always refer to this whole, and draw conclusions only to its effects in the whole.*" (Emphasis added, Bernard, 1865)

It cannot be overstated that the whole *is* greater than the sum of the parts!

The grand challenge faced by contemporary physiology in this post-genomic era as first described in 2010 (Billman, 2010) remains how to integrate and to translate this deluge of information obtained *in vitro* into a coherent understanding of function *in vivo*. Although a machine may consist of many parts, the parts in isolation do not make the machine. Anyone who has tried to assemble a child's bicycle on Christmas Eve can testify that the parts do not a machine make. In an analogous fashion, while organisms are made of molecules, molecules are not organisms. The concept of one gene, one protein, one function is woefully inadequate to explain the dazzling complexity and startling beauty of the living organism – the intricate dance of homeostatic mechanisms necessary for a "free and independent life." A sequence of base pairs in the DNA molecule can no more explain the complexities of life than a series of 1s and 0s on a compact disc recording can explain the emotional response to music (Noble, 2006). Man and other organisms are not mere vehicles for the perpetuation of genes, selfish or otherwise. The days for reductionist deconstruction are numbered; more holistic and integrated systems approaches are required to put Humpty Dumpty back together again. It is time for physiologist to return to their roots and consider the organism as a whole as advocated by Claude Bernard.

A second, and by no means less important, challenge will be to train the next generation of scholars to perform the integrative studies in intact preparations (whole animals or organs) that are the pre-requisite for clinical applications. Unfortunately, there has been a progressive decline in the number of integrative physiology training programs, resulting in a paucity of individuals with the skill sets necessary for whole animal *in vivo* experimentation. The problem is exacerbated by the renaming or actual elimination of Departments of Physiology within Colleges of Medicine. It currently is fashionable for physiology departments to rechristen themselves as "Departments of Molecular Biology/Physiology." With tongue firmly in cheek, one wonders if Departments of Atomic Physiology will be soon in the offing.

With the increasing emphasis on molecular and genetic approaches, it is not unusual to find members of physiology departments who have not even taken an introductory course in physiology. This is, indeed, a shame as much of the excitement for physiology as an intellectual discipline can best be encountered in the student lab. Nothing can replace the hands-on learning nor instill a better appreciation for the concept of homeostasis than performing these classic physiology experiments. In the student lab, one can go beyond the dry textbook description of physiological principles and see them in action. The student can experience, first hand, the same excitement and sense of wonder that the earlier investigators must have had when they first examined skeletal muscle-nerve function in frogs, saw the clearance of dye in the easily visible glomeruli in the necturus (mudpuppy), or pondered the mysteries of cardiopulmonary regulation in mammals (rat, rabbit or dogs). Thus, it very much remains an open question as to whether a sufficient number of suitably trained investigators will be available to meet the grand challenge: to integrate function from molecules to intact organisms.

SUMMARY

Our understanding of physiological regulation has evolved over time from the Greek idea concerning the balance between the body humors, through Claude Bernard's "milieu intérieur" to Walter Cannon's formulation of the concept of homeostasis and the application of control theory (feedback regulation) to explain how a constant internal environment is achieved. Homeostasis has become the central unifying concept of physiology and is defined as a self-regulating process by which a living organism can maintain internal stability while adjusting to changing external conditions. Homeostasis is not static and unvarying; it is a dynamic process that can change internal conditions as required to survive external challenges. This is made clear by the care Cannon used when coining the word homeostasis. He deliberately selected Greek words that when, combined, meant "staying similar" rather than "staying the same" to emphasize that internal conditions could vary yet still produce stability (within a range of values rather than a single value). Thus, homeostasis does not mean "stagnation." It is also important to note that homeostatic regulation is not merely the product of a single negative feedback cycle but reflects the complex interaction of multiple feedback systems that can be modified by higher control centers. This hierarchical control and feedback redundancy produces both a finer level of control and a greater flexibility that enables the organism to adapt to changing environmental conditions. The health and vitality of the organism can be said to be the end result of homeostatic regulation of the internal environment; an understanding of normal physiology is not possible without an appreciation of this concept. Conversely, it follows that disruption of homeostatic mechanisms is what leads to disease, and effective therapy must be directed toward re-establishing these homeostatic conditions, working with rather than against nature.

AUTHOR CONTRIBUTIONS

GB prepared all aspects of this review article.

ACKNOWLEDGMENTS

As previously stated, portions of the material presented in this essay were previously published as Chapter 10 in "Handbook of Systems and Complexity in Health" (Billman, 2013) and are reproduced (with modification and updates) with the permission of the publisher.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

SPECIALTY GRAND CHALLENGE
published: 26 March 2021
doi: 10.3389/fphys.2021.662878

New Challenges in Systems Biology: Understanding the Holobiont

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Keywords: systems biology, holobiont, evolution, complex ecosystem, dynamics, challenge

More than a century ago, C. Darwin formulated the Theory of Evolution based on Natural Selection without knowing how information is transmitted from one generation to another. In Darwin's theory, the concept of *individual* is of fundamental importance: Individuals mutate, and advantageous mutations that improve adaptation to the environment are passed on to the next generations, perpetuating an improvement cycle. Now we know that mutations occur in the genomes of single individuals, and those mutations, if beneficial (or neutral), can be passed on to the next generations. However, in the Theory of Evolution formulated by Darwin and accepted for more than a century, the concept of individual is taken for granted: an individual is a single entity that contains (in its genome) all the necessary information to generate organisms with similar (or identical) phenotypes. However, the very concept of "individual," on which Darwin's theory relies, has recently been challenged (Bordenstein and Theis, 2015; Suárez and Stenzel, 2020).

Today, we know that every multicellular organism is an ecological system. When such organisms emerged on Earth several millions of years ago, the planet was already crowded with microbial life. Consequently, the emergence and evolution of plants and animals were not only carried out in the presence of microbes but, in many cases, such evolution was only possible because of these microorganisms (Zilber-Rosenberg and Rosenberg, 2008; Alegado et al., 2012; Carpenter, 2012; Gaulke et al., 2016). Recent studies have unequivocally shown that there is a great variety of microbes living in plant and animal hosts, the totality of which is known as the host's *microbiota.* For humans, it has been estimated that the number of bacterial cells inhabiting a human body is comparable with the number of body cells (Abbot, 2016; Sender et al., 2016), and that the microbiota strongly interacts with its host, regulating important metabolic functions at all levels, including the genetic level (Bates et al., 2006; Wang et al., 2018).

The human microbiota helps in the development of the immune system, the fortification of bones, the digestion of food, the regeneration of skin, and affects many other essential metabolic functions. Furthermore, there is a strong correlation between the microbiota's composition and the occurrence of complex diseases such as obesity, diabetes, cancer, metabolic syndrome, inflammatory bowel disease, allergies to gluten and lactose, and even cognitive and neurological disorders such as autism and schizophrenia (Wang et al., 2017, 2018; Fan and Pedersen, 2021). Multicellular organisms have coevolved with microbes and strongly depend on them (even bacteria have their microbiota, which consists of viruses). Therefore, the question arises: what is an individual?

To emphasize that every individual is, in fact, a complex ecosystem, Rosenberg and his collaborators (Theis et al., 2016; Roughgarden et al., 2018) have proposed to use the term *holobiont* [first introduced by Lynn Margulis in a different context (Guerrero et al., 2013)] to refer to the host organism together with its microbiota. The holobiont is more than just an aggregate of cells that live together, as all cells in an organism carry the same genetic information in all organs. By contrast, in a holobiont, cells with different genetic compositions (even from different kingdoms), live together, interact, and exhibit complex dynamical behaviors. Imbalances in the holobiont ecosystem may

OPEN ACCESS

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Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Physiology

Received: *01 February 2021*
Accepted: *22 February 2021*
Published: *26 March 2021*

Citation:
Aldana M and Robeva R (2021) New Challenges in Systems Biology: Understanding the Holobiont. Front. Physiol. 12:662878. doi: 10.3389/fphys.2021.662878

lead to *dysbiosis,* a medical condition that may cause unwanted symptoms and, as mentioned above, has been associated with the onset of serious diseases. For a long time, predisposition to such diseases was attributed only to particularities in the genetic material of the host organism. However, if the microbiota's composition is strongly correlated with a complex disease, say obesity, it is possible that the microbiota that favors the occurrence of that disease is being transmitted from mother to offspring (Veigl et al., 2019). If that were the case, then the inheritance of phenotypes could be attributed not only to the genetic composition of the host organism but also to the transmission of microbes across generations.

Such connections would potentially challenge one of the central dogmas in the current Theory of Evolution, making Lamark's type of inheritance possible (Rosenberg et al., 2009). Indeed, there is strong evidence suggesting that. For instance, we know that obesity, diabetes, or breast cancer are inherited with high probability, as shown by familial studies. However, genetic markers for these diseases are yet to be discovered (a problem known as "*the missing heritability*") (Manolio et al., 2009; Eichler et al., 2010; Génin, 2019). One possible explanation may hide in plain sight: our efforts to answer these questions are focused on looking for genetic markers only in the human genome (namely just in a tree of the entire forest), while we now know that the human microbiota is strongly correlated with the emergence of complex diseases. Therefore, when looking for genetic signatures of these diseases we should be looking at the entire forest; that is, consider not only the human genome but also the totality of genomes of all organisms in the microbiota—the *microbiome* (Sandoval-Motta et al., 2017).

Treatments for some illnesses, like the irritable bowel syndrome, that aim to rebalance the microbiota by transplanting fecal matter from a healthy person to a sick person (therapy known as "fecal transplant" or "bacteriotherapy") date back to ancient China (De Groot et al., 2017). More recent studies (Woodworth et al., 2019) have shown that this approach is effective in reducing intestinal colonization with antibiotic-resistant bacteria, even though we may not yet fully understand the mechanism at the molecular or genetic levels. This suggests that transplanting bacteria from healthy to sick people may also work for other complex diseases such as obesity, cancer, or autism. The answer is yet to be discovered.

The strong symbiotic interactions between bacterial communities and their hosts (including humans) within the holobiont, were discovered only recently with the development of high-throughput sequencing techniques (Visscher et al., 2017). Most bacteria in the microbiota cannot be cultivated outside their host organism—let by themselves, these bacterial communities establish interactions dominated by competition, resulting in the dominance of one bacterial strain. It is the host that regulates these otherwise competing interactions, allowing different bacterial communities to inhabit the same organism (Foster and Bell, 2012; Coyte et al., 2015). As already mentioned above, bacteria in turn help the host in carrying out many different metabolic functions. High-throughput sequencing techniques provide a way to reveal the structure of complex ecosystems hidden inside the host and unveil those symbiotic interactions (Wang and Jia, 2016). The host, in turn, influences the composition of the microbiota. In the case of humans, the type of food we eat exerts an influence. A concrete example can be found in some bacteria living in the gut which transform carbohydrates into serotonin, a neurotransmitter associated with happiness (Stasi et al., 2019). Therefore, eating carbohydrates makes us happy, which creates a positive feed-forward cycle between us and gut bacteria that can lead to obesity and diabetes (It is still a matter of debate how the serotonin produced in the gut can cross the blood-brain barrier and reach the neurons in the brain).

It has been almost a decade since the pioneering work by Turnbaugh and his collaborators, who demonstrated that the bacteria in the human gut can determine important phenotypic traits (obesity) in mice (Turnbaugh et al., 2006). Since then, we have learned a lot about the symbiotic relationships between microbes and multicellular organisms. Mathematical models have also been deployed to investigate holobiont selection as an evolutionary force (Huitzil et al., 2018; Roughgarden, 2019). But we have only scratched the surface. There are many problems and questions that remain unsolved. In our opinion, one of the greatest challenges that we face for the twenty-first century regarding Systems Biology is to develop mathematical and computational models that help us understand the holobiont as a complex ecosystem. How and why such strong symbiotic relationships between bacteria and multicellular organisms have appeared throughout evolution? Did they appear just because it was possible? If not, what evolutionary advantages emerge from these symbiotic interactions? Will the concept of "individual" in evolutionary theories need to be reformulated (or replaced) to take into account the holobiont as a complex ecosystem subject to selection? What complex diseases could be cured by altering the microbiota's composition of the patient? Are there phenotypes that can be "inherited" through the microbiota and not through parental DNA? Is it possible to engineer a "healthy microbiota" or a microbiota that favors the emergence of desired phenotypes in the host organism?

The list of major challenges and unanswered questions is already long. And as we unravel some of these questions in the future, the list will get even longer. The Systems Biology community around the world faces a significant challenge—we need experimental methods, mathematical models, and computational approaches that combine the best available data from genomics, metabolomics, and proteomics with existing knowledge in the life sciences to help us understand the evolution, dynamics, and behavior of holobionts not as individuals, but as complex ecosystems.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

RR was partially supported by the Karl E. Peace Fellowship at Randolph-Macon College.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

SPECIALTY GRAND CHALLENGE
published: 12 April 2021
doi: 10.3389/fphys.2021.674951

The Future of Gastrointestinal Physiology: 2020 and Beyond

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Keywords: gastrointestinal, gastroenterology, gut, physiology, microbiome

GASTROINTESTINAL SCIENCES

There have been dramatic advances in gastrointestinal sciences over the past 10 years since the inauguration of the Frontiers in Gastrointestinal Sciences. In 2010, I suggested that the field would rapidly embrace the findings about the role of the gut microbiome in physiologic homeostasis as well as disorders of homeostasis; and that there would be a significant advance in our understanding of gut sensors and their connection to neurohumoral pathways mediating systemic responses (Pandol, 2010). Importantly, dietary nutrients and metabolic products of the dietary nutrients could play an important role in regulating systemic responses through the gut sensors. Frontiers has contributed importantly and broadly to advances in these areas and has provided a multidisciplinary platform for integrating the sciences involved. The specifics of cellular physiology and pathways are critical components to our mechanistic understanding of physiology at multiple organs and tissues, the functions of the gastrointestinal tract and its effects on systemic physiology are known to be regulated by what is going on in the gut lumen from dietary constituents ingested, the effects of ingested nutrients on the gut microbiome and the multiple pathways stimulated at the gut wall that have profound and widespread effects on the entire body. Thus, what happens in the lumen of the gastrointestinal tract can have broad effects in homeostasis and disease.

Regarding the gut microbiome, I thought that interdisciplinary scientific approaches would start to reveal the interplays between diet, the gut microbiome epithelial function and immune functions, and systemic metabolism. Disorders of one or more participants in this interplay would likely underlie some of the most common and devastating medical problems of our time including the metabolic syndrome (obesity, diabetes mellitus, hypertension and liver, and vascular diseases), inflammatory gastrointestinal diseases and predisposition to several cancers. This call was followed by an inspirational review Frontiers published by Grenham et al. (2011) providing observations of the role of the gut microbiome in intestinal function including proliferation and differentiation of the epithelium and the development of the immune system and activation of neural pathways. Grenham et al. (2011) also outlined hypotheses about the role of the gut microbiome and the central nervous system (CNS) indicating that CNS stress may cause gut disorders of such as irritable bowel syndrome through effects on the gut microbiome; and that disorders in the gut microbiome could have effects on the CNS. Another highly cited review by Greer and O'Keefe (2011) reviewed evidence suggesting that the westernized diet, the overuse of antibiotics and decreased breast feeding through alterations in the gut microbiota promotes the inflammatory potential in the bowel creating increased risk for inflammatory diseases and cancer of the bowel in those with a genetic predisposition. The review further points out the increased risk of these disease is associated with the suppression of microbial fermentation and production of butyrate which is anti-inflammatory and anti-proliferative (Greer and O'Keefe, 2011).

More recently, Bliss and Whiteside (2018) provided a framework for understand the complex relationship between the Western diet, the gut microbiome, lumenal sensors on gut enteroendocrine cells axis via local, paracrine and/or endocrine mechanisms involving a variety of gut-derived peptides produced from enteroendocrine cells. The sensors on the strategically located gut enteroendocrine cells interacting with meal nutrients and microbial metabolites activate intracellular signaling systems to release hormones including cholecystokinin (CCK), glucagon-like

OPEN ACCESS

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Specialty section:
This article was submitted to Gastrointestinal Sciences, a section of the journal Frontiers in Physiology

Received: *02 March 2021*
Accepted: *09 March 2021*
Published: *12 April 2021*

Citation:
Pandol S (2021) The Future of Gastrointestinal Physiology: 2020 and Beyond. Front. Physiol. 12:674951. doi: 10.3389/fphys.2021.674951

peptide 1 (GLP-1), and peptide YY (PYY). We know that these hormones have regulator effects on a large number gastrointestinal organs as well as the CNS. Some of these effects include slowing of gastric emptying, secretion of biliary and pancreatic enzymes, promotion of insulin secretion and inhibition of feeding. Not only do CCK, GLP-1, and PYY have hormonal effects but they have paracrine effects on neural sensory pathways carrying information in the enteric nervous system to the CNS and then to motor pathways having further regulatory effects. Finally, recent findings show that the enteroendocrine cells have neural properties and directly synapse with the neurons of the enteric nervous system (Liddle, 2019). In combination, these findings indicate that nutrients, and microbial metabolic products not only have local effects on epithelial function but have widespread effects systemically through the immune system, hormone secretion and neural pathways that connect to the CNS.

Since these inaugural articles there has an avalanche of scientific papers on the gut microbiome in the literature. A PubMed search shows that in 2010 there were < 100 papers with the topic of gut microbiome while in 2020 the number was just under 7,000- a near 700 times increase. Frontiers in Gastrointestinal Sciences has played a significant role in publishing important papers in the emerging field. These include papers on the role of the gut microbiome in both alcoholic (Su et al., 2016; Yue et al., 2020; Zheng et al., 2020) and non-alcoholic (Bian et al., 2017; Porras et al., 2018; Zheng et al., 2018) liver disease; the role of sulfur metabolism by the microbiome on gut sensory signaling, colonic inflammation and cancer; (Carbonero et al., 2012; Bala et al., 2014; Guo et al., 2016; Burgueño et al., 2019; Fei et al., 2019; Song et al., 2019; Ren et al., 2020) glucose homeostasis and type 2 diabetes; (Lê et al., 2012; Su et al., 2016) tuberculosis (Luo et al., 2017) mood and behavior and Gulf war illness; (Grenham et al., 2011; Kimono et al., 2019) pulmonary tuberculosis; (Luo et al., 2017) and autoimmune pancreatitis (Haruta et al., 2012). Other papers provided information on the effects of xenobiotics, probiotics, vitamins, and medication affect the gut microbiome and physiology (Yang et al., 2015; Su et al., 2016; Atashgahi et al., 2018; Li et al., 2018; Zheng et al., 2018; Chang et al., 2019; Fei et al., 2019; Guan et al., 2019; Song et al., 2019; Xu et al., 2019; Zhu et al., 2020) while some addressed the interplay between endogenous processes and the gut microbiome including bile acids and cholesterol metabolism, adrenergic input and gut epithelial responses (Yang et al., 2017; Burgueño et al., 2019; Molinero et al., 2019).

This discussion is not meant to suggest that important observations in gastrointestinal sciences outside of the microbiome are not highly considered by Frontiers. On the contrary, the discussion is meant to suggest that the expansion of the field will occur with greater mechanistic understanding of impact of the microenvironment of the gut organs including dietary constituents, xenobiotics and metabolic products of the microbiome have on the secretory, absorptive, elimination, motility, endocrine, neural and immune functions of the gastrointestinal system. As the field progresses greater use of co-culture of *in vitro* and *ex vivo* models including organ on a chip technology with microorganisms; (Pearce et al., 2018) and use of germ-free animal models (Kennedy et al., 2018) to selectively introduce a microbiome under investigation will be encouraged. This article is meant to encourage continued multidisciplinary approaches to further the progress in gastrointestinal sciences so that new understanding can be translated to benefit human health.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SPECIALTY GRAND CHALLENGE
published: 15 May 2020
doi: 10.3389/fphys.2020.00489

Integrative Physiology: Update to the Grand Challenge 2020

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Keywords: physiology, omics, grand challenge, research translation, research opportunities

INTRODUCTION

After a decade has elapsed since I wrote the grand challenge for integrative physiology in 2010, it is fitting to not only reflect on how well we have progressed in this journey but also to consider the future challenges and opportunities that arise. It is also of interest to see how various physiology journals have been fairing in terms of impact factors and published articles. From a sample of eight prestigious physiology journals, it appears that the field of physiology itself has remained strong with no change or trend in average impact factor over this time (**Figure 1**). From a sample of 8 prominent physiology journals, the median impact factor of 4.5 in 2010 has increased to 5.4 some 9 years later and the average number of articles per journal has doubled from 182 to 373 (**Figure 1**). The standout improvers are Frontiers in Physiology that increased the number of published articles from 59 in 2010 to 1984 in 9 years and also Acta Physiologica that improved its impact factor nearly 2-fold from 3.1 in 2010 and to 5.9 in 2018. Interestingly, open access and general science journal PLOS ONE has declined in impact from 4.4 to 2.8 while increasing articles from 7000 to 18000. Journal of Neuroscience impact factor has also declined slightly from 7.2 to 6.0 as has the number of articles dropped from 1,700 to 800 in the same 9-year period. While these are anecdotal examples from other fields and limited evaluation in terms of quality and quantity, they do suggest that physiology at large has been doing quite well as a field over the last 10 years at least maintaining and, in some cases, improving.

The Impact of "Omics" on Physiology

In my 2010 article discussing the grand challenge for integrative physiology, I emphasized new directions that had confronted physiologists related in particular to the genetic and "omics" revolution which had transformed biomedical research in the preceding years. The important question is whether we have adopted the challenge of the new information and technology or simply continued to generate research outcomes along our traditional lines. The large-scale investment in these platforms challenged traditional areas of endeavor such as physiology and has diverted the focus of biomedical research. However, there are many opportunities now and, on the horizon, to utilize the marvelous new technological developments which will hopefully spark physiologists' interest and create collaborative productivity. New models with tissue specific overexpression, constitutively active receptors and dominant negative genetics have been well-utilized and continue to expand our physiological understanding of complex systems and how they are affected by disease. There are also wonderful new technologies such as CRISPR/Cas9 for global, conditional and targeted gene editing and now *in-vivo* CRISPR/Cas9 gene editing. There have been 16 studies in Frontiers in Physiology since 2017 that have utilized this technology and interestingly none before that year.

High throughput genomics, proteomics, metabolomics, transcriptomics, nutrigenomics and more recently analysis of the human microbiome has given us a huge array of information that must also be contextualized within its physiological setting. Novel targets that are identified can be coupled with high throughput cellular screens to discover new therapies with physiological

OPEN ACCESS

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Specialty section:
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology

Received: *07 March 2020*
Accepted: *21 April 2020*
Published: *15 May 2020*

Citation:
Head GA (2020) Integrative Physiology: Update to the Grand Challenge 2020. Front. Physiol. 11:489. doi: 10.3389/fphys.2020.00489

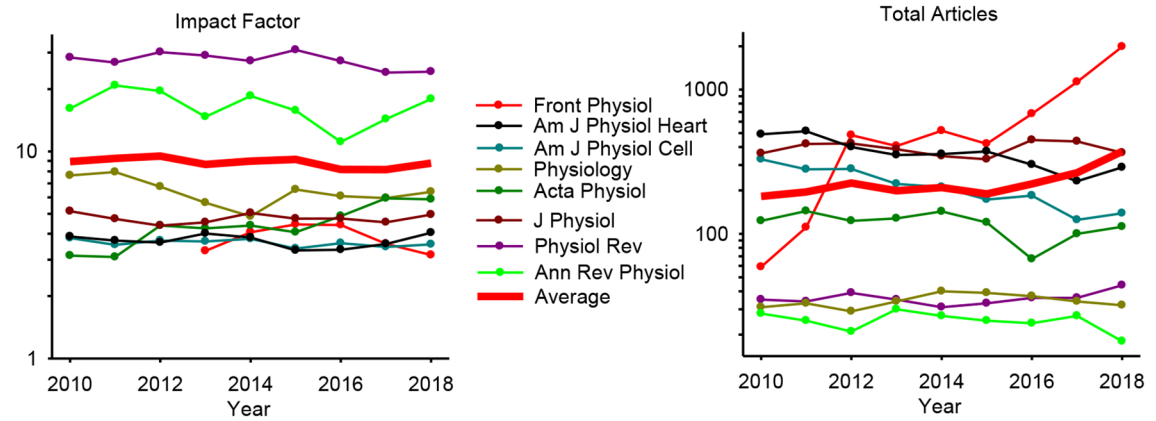

FIGURE 1 | Year by year impact factor (**Left**) and total articles (**Right**) in 8 selected high-level physiology journals. The average trend line is shown in thick red.

regulatory potential. The discoveries of intracellular signaling, second messengers and regulatory influences such as post transcriptional modulation have taken our understanding of physiological processes to a new level. It is clear that physiologists that can integrate this information have a critical role in both the preclinical and clinical phases of discovery. However, the challenge will be to transform our thinking to embrace these new and quite marvelous opportunities. It is worth noting that in 2011 only 21 articles were published in Frontiers in Physiology that mention "omics" compared to over two thousand in 2019 which is a 10-fold growth. Interestingly, the omics field as determined by a PubMed search, has grown by 5-fold in that same period. Thus, we might conclude from this perhaps rudimentary analysis that physiologists at least in the Frontiers in Physiology journal, have embraced this new challenge with vigor. The major limitation however, is the amount of resources required to perform phenotypic analysis on all these new models and genetic variants. I suspect that we are not training and supporting sufficient physiologists to really cope with this wave.

Microarray and GWAS Studies

A major advance in the hypertension field has been to use genetic risk scores to find genetic loci that contribute to high blood pressure. They combine the cardiovascular risk associated with variations in multiple genetic loci across the genome using genome-wide association study (GWAS). The major advantage of obtaining genetic risk scores using this method is that individual gene variants are less important and therefore the score is less influenced by imperfect linkages (Ehret, 2010). By and large however, such approaches have been disappointing as they explain a few percent of the overall cardiovascular risk (Head, 2016). The problems with such human studies are that the associations do not differentiate between genes that are changed due to high blood pressure and those that are causing it. Also, the genetic associations may well change as the course of the disease develops from the initiating phase to the structural and other changes that occur in vessels and the heart over many years (Ehret, 2010).

Experimental animal models of different diseases can be used to illuminate the mechanisms within tissue and systems that are not accessible in human studies. There are a number of rat and mouse strains for example that have been bred or genetically manipulated to develop high blood pressure. Strains such as the spontaneously hypertensive rat (SHR) and Schlager BPH mouse developed in the 1960's and 1970's, respectively have been widely used (Okamoto and Aoki, 1963; Schlager, 1974; Jackson et al., 2019). Studies from our laboratory suggested that the BPH mice had a neurogenic form of hypertension involving a much greater contribution of the sympathetic nervous system (SNS) (Davern et al., 2009). Marques and colleagues examined the hypothalamus of young and old BPH mice and compared them to the normotensive control BPN strain using gene array in 2011. While there were a number of genes associated with the development of hypertension, an unusual up and down pattern of expression of specific subunits of the $GABA_A$ receptor was discovered (Marques et al., 2011a,b). Notably there was a lack of message for δ, α4 and β2 subunits particularly at 6 weeks of age when the hypertension was evident. To test the hypothesis that the overactive SNS was due to lack of $GABA_A$ inhibitory signal in pre-sympathetic pathways, a $GABA_A$ allosteric modulator benzodiazepine was administered chronically which had no effect on the blood pressure in the hypertensive mice but lowered blood pressure in the normal mice (Davern et al., 2014). This indicated that there was indeed a difference in the $GABA_A$ receptors influencing blood pressure. By contrast, the neurosteroid allopregnanolone which is also an allosteric and expression modulator of $GABA_A$ receptors had no effect in the normal mice but lowered blood pressure in the hypertensive mice (Stevenson et al., 2017). Importantly, the hypotensive action was associated with a restoration of the δ, α4 and β2 subunits expression in the hypothalamus and amygdala (Stevenson et al., 2017). Thus, a new potential therapeutic to treat hypertension has been revealed from the initial discovery using an exploratory microarray analysis (Head et al., 2019).

Importantly, this therapy would target the SNS reactivity to stress which is not a mechanism that is targeted by current therapy modalities (Head et al., 2019).

RNA-Sequencing

The development of RNA-sequencing has been a major step forward since it uses next generation sequencing to determine the transcriptome profile of any particular experimental or clinical scenario to reveal novel affected transcripts. The technique has the advantage over microarray in that it is limited to known genes. A recent review by Adeola et al. explored the implications of "omics" technology in the study of clock genes (circadiOmics) which encompasses the use of genomics, transcriptomics, proteomics and metabolomics (Adeola et al., 2019). In an excellent example using both RNA-seq and DNA arrays, Zhang and colleagues found that 43% of all genes were influenced by circadian rhythms (Zhang et al., 2014). The authors suggested that their study "highlights critical, systemic, and surprising roles of the mammalian circadian clock and provides a blueprint for advancement in chronotherapy."

A recent advance has enabled RNA sequencing to be attributed to cells thus we can find populations of different cell types in a tissue with characteristic expression and in doing so, we can reveal rare cell populations and discover important regulatory relationships between genes. Thus, apparently histologically similar adjacent cells can have quite different expression profiles. Steven Potter has written an excellent review of single cell sequencing in development, physiology and disease (Potter, 2018). One example of note that piqued my interest in the capabilities of single cell RNA sequencing comes from Chen and colleagues who used this technique to reveal a much more complex cell diversity in the mouse hypothalamus than previously thought (Chen et al., 2017). They not only found the expected known neuropeptide and peptide combination containing neurons, they also found previously undescribed cell groups. Importantly, they went on to show that food deprivation affected the transcriptome of 7 of the 34 subtypes and in doing so uncovered cell types not previously associated with food intake (Chen et al., 2017). Thus, by using relatively simple physiological challenges one can reveal which cells respond and in what way they change their expression profile.

MicroRNA

MicroRNAs (miRNA) are small non-coding RNAs that interact with the 3' untranslated region of specific RNAs to induce degradation (O'Brien et al., 2018). They can also induce translational repression. They are considered to be master regulators of gene expression and have been used as biomarkers since they are relatively stable and can be found in plasma (Roser et al., 2018). While their discovery was in 1993, they have increasingly been the focus of researchers interested in how gene expression is regulated during health and disease (Bhaskaran and Mohan, 2014). Importantly, discoveries in miRNA gene regulation offer the opportunity for novel therapy since mimics and inhibitors are now available and have been used *in vivo* (Bhaskaran and Mohan, 2014). There is one word of caution however, since the transfection may not exactly mimic the endogenous function (Jin et al., 2015). High concentrations may have non-specific consequences and even transfection at physiological concentrations may not induce changes in gene expression (Jin et al., 2015).

Marques and colleagues examined the differential expression of miRNA between kidneys of patients with high and normal blood pressure and found that miRNA-181a suppresses renin expression (Marques et al., 2011c). Renin expression was 6-fold higher in hypertensive kidneys and miRNA-181a levels 6-fold lower. *In vitro* studies showed that this miRNA bound to renin and regulated renin expression (Marques et al., 2011c). Interestingly, a similar renin-miRNA-181a pattern was discovered in the kidneys of the BPH hypertensive mouse where higher levels of renin were observed when levels of miRNA-181a were lowest (Jackson et al., 2013). This occurred at night when the mouse was most active and the SNS activity was highest (Jackson et al., 2013). During the day, there was no difference between the normotensive and hypertensive strains in either renin expression in the kidney or in miRNA-181a. One possibility for this difference between day and night might be that the miRNA is under the influence of the SNS and possibly circadian clock genes. Indeed, renal nerve denervation completely abolished the circadian differences in renin expression in the kidney, supporting this possibility.

We should not only consider 24-h patterns of expression but also longer periods such as might occur with aging. Yao and colleagues examined old and young human atrial tissue to identify how microRNA, genes and miRNA-mRNA interactions change with aging. They found 7 miRNA's, 42 genes and 114 pairs on miRNA-mRNA interactions differentially expressed (Yao et al., 2019). These types of studies are just the beginning to characterize how we age "genetically" and how these processes might be altered.

It is also of great interest that short-term interventions can alter miRNA levels. Yin and colleagues evaluated such a time-course in muscle-specific microRNA (miRNA) after rats ran uphill or downhill for 90 min (Yin et al., 2019). Interestingly, the miRNAs of interest were not affected by running uphill but were all increased after running downhill. Clearly some miRNAs are able to be regulated within the very short time frame of hours while others were induced after 48 h. These characteristics, once revealed for not only this type of intervention, but also other conditions such as stroke or myocardial infarction may be useful biomarkers and lead to a better understanding of mechanisms.

CONCLUSION

In this review, I have touched on some of the opportunities that developments in omics and genetic technology have offered physiologists to explore. Clearly this is happening which is very pleasing to discover. I have highlighted only a few of the new techniques and examples that are now available. Really this is an extraordinary time in biomedical research. The first gene editing in humans using CRISPR/Cas9

for example is happening now (NCT03872479). The grand challenge as it was in 2010, will be for physiologists to be the translational link between the discoveries and the clinical trials. Importantly, we bring new insights and opportunities to our clinicians and pharmaceutical scientists. We must continue to build strong collaborations with our omics colleagues and utilize the new approaches to focus on mechanisms and regulatory functions that govern our physiological state and our health.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SPECIALTY GRAND CHALLENGE
published: 10 June 2021
doi: 10.3389/fphys.2021.706061

Developmental Physiology: Grand Challenges

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Keywords: DOHaD, developmental plasticity, climate change, toxicology, epigenetics, heterokairy, allometry, animal models

INTRODUCTION

The field of Developmental Physiology faces both great opportunities and significant challenges in the years ahead. While this provides "job security" for new investigators in this area, it also requires delineating some of the major challenges to maximize progress. I suggest that pressing challenges in Developmental Physiology fall into three categories: demonstrating broad relevance, promoting conceptual advances and improving experimental approaches.

CHALLENGE: DEMONSTRATING BROAD RELEVANCE

Developmental Origins of Health and Disease

The developmental origins of health and disease (DOHaD) encapsulates how environmental stressors in early development can lead to subsequent health implications in adult humans (**Figure 1**). DOHaD is now influencing many fields of research (Suzuki, 2018), including epidemiology (Heindel et al., 2017), epigenetics (Safi-Stibler and Gabory, 2020), specific pathophysiologies (Arima and Fukuoka, 2020; Briana and Malamitsi-Puchner, 2020) and policy and public health (Loi et al., 2013), to name but a few such fields. Among the many challenges for human-oriented studies in DOHaD is translating what we learn from developmental studies into actionable clinical and public health practices (Hanson et al., 2019). What is increasingly understood is how early developmental trajectories can be programmed by early developmental events. Yet, adult disease prevention through intervention in early development in many instances lags behind the more common (and far more challenging, not to mention expensive) practice of treating diseases in adults arising from, as but one example, maternal or neonatal nutrition (Baird et al., 2017). In basic animal physiology, the consequences of early life conditions for adult experimental animals are not generally appreciated (or are ignored), judging from the consistent lack of any mention of rearing conditions in published physiology articles using adult animal models. Recognizing how early life conditions alter adult physiology remains a challenge for the field.

Developmental Physiology and Climate Change

Great effort is being expended to determine the effects of projected climate change on individual organisms all the way up to the ecosystems they inhabit. All too often, however, studies singularly involve adults of a species. Far less studied are effects in developing animals of temperature change, ocean acidification and other climate change-related phenomena. This exposes a truism: there will be no adults of a species to experience climate change effects if the species' offspring do not develop correctly (or at all) in the first place! Indeed, climate change presents numerous possible stressors that could affect development in a similar manner to DOHaD-related stressors during the developmental process (Kingsolver and Buckley, 2020; Sanger, 2021). One of the challenges for

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Specialty section:
This article was submitted to Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *06 May 2021*
Accepted: *10 May 2021*
Published: *10 June 2021*

Citation:
Burggren W (2021) Developmental Physiology: Grand Challenges. Front. Physiol. 12:706061. doi: 10.3389/fphys.2021.706061

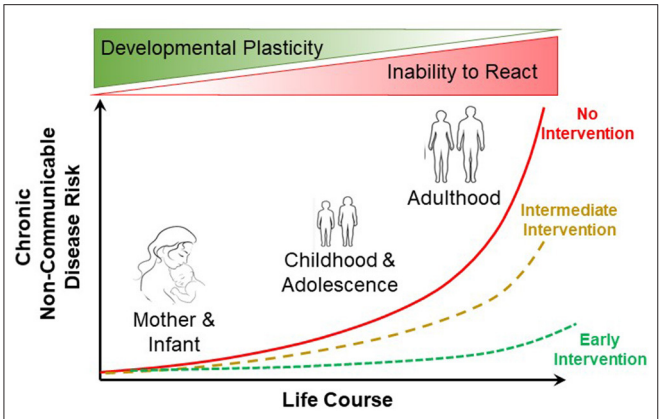

FIGURE 1 | Influence of early life course intervention on the risk of non-communicable disease in humans. Early intervention (e.g., improved maternal and neonatal nutrition) can result in greatly reduced risk of non-communicable disease in adulthood. Modified from Baird et al. (2017).

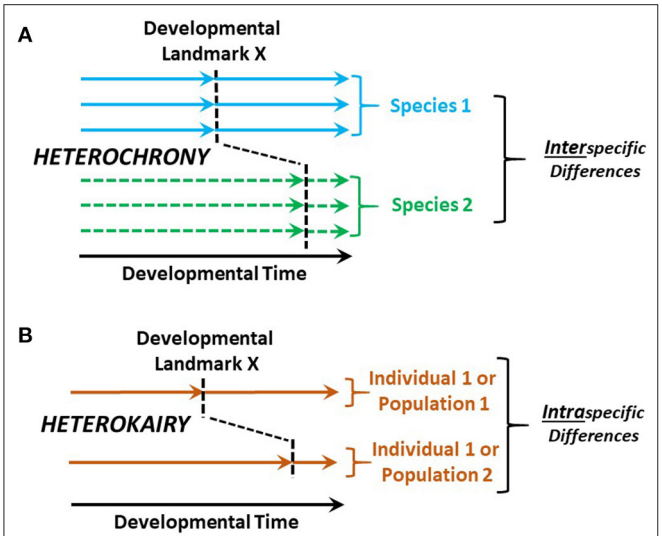

FIGURE 2 | Heterochrony compared with heterokairy. **(A)** Heterochrony compares the timing of developmental landmark between a species of interest and the ancestral species. **(B)** In contrast, heterokairy compares the developmental landmarks of two different individuals or populations within a single species. Typically, the developmental landmarks are anatomical—e.g., the appearance of gill arches, cardiac valves or nephrons. However, these concepts apply equally well to physiological developmental landmarks such as the onset of gill ventilation, the onset of anterograde blood flow through the heart, or the first production of urine.

Developmental Physiology, then, is to emphasize and analyze effects of climate change on early life stages and how this ultimately influences species fitness.

A Deeper Understanding of Toxicological Effects During Development

The early life stage approach advocated above has been exploited in toxicology and the study of environmental contamination. A prime example of this is the April 2010 DeepWater Horizon oil spill in the Gulf of Mexico, where considerable focus has been on the effects of oil exposure in developing fish and birds. What emerges is that early life stages have often higher vulnerability to environmental toxicants than at later stages (Incardona and Scholz, 2018; Pasparakis et al., 2019). Yet, what is also becoming clear is that organisms can develop considerable resilience to toxicants that can transfer to their offspring through non-genetic mechanisms (Vandegehuchte and Janssen, 2014; Kishimoto et al., 2017; Bautista and Burggren, 2019). A challenge, then is to understand the limits and mechanisms underlying inheritance of this toxicant resistance and how this might alter both science and policy involving environmental contamination.

CHALLENGE: PROMOTING CONCEPTUAL ADVANCES

Expanding the Role of Epigenetics

Mendelian genetics is increasingly inadequate in explaining key developmental phenomena ranging from misdirected gene expression resulting in adult disease states to non-genetically inheritance of modified phenotypes. We now know that, typically, the modification of DNA expression in germ or somatic cells alike that produces modified phenotypes involves "readers" that assess the state of epigenetic markers on genes. Subsequently, "writers" and "erasers" modify the state of epigenetic markers according to the prevailing environment and thus modify expression of genes that these markers regulate. The result is altered phenotypes within or across generations (Walker and Burggren, 2020; Wan et al., 2021; Wei and He, 2021). Essentially, developmental phenotypic plasticity, one of the basic principles of developmental physiology (Burggren, 2020a) and discussed above in the context of DOHaD, derives largely from the writing and erasing of epigenetic markers. A challenge for developmental physiologists is to integrate, from molecular to organismal level, the mechanisms and outcomes associated with epigenetic reading, writing and erasing and the associated changes in gene expression, both within and across generations.

Physiological Heterochrony and Heterokairy

Heterochrony is the change in timing of developmental landmarks in a species compared to its ancestral species (Keyte and Smith, 2014). As a companion concept, heterokairy focuses on the intraspecific change in timing of developmental landmarks in an individual or a population (**Figure 2**). Like heterochrony, the concept of heterokairy is proving similarly useful in understanding developmental processes (Spicer and Burggren, 2003; Rundle and Spicer, 2016; Spicer et al., 2018). However, both of these concepts are typically employed to consider anatomical development with typically less focus on physiological processes. Yet, both concepts also equally apply to physiological development. The challenge for developmental physiologists, then, is to more deeply investigate

heterochrony and especially heterokairy, allowing important insights into phenomena such as developmental phenotypic plasticity and the contributions of physiological processes to fitness during development.

Allometry and Development

The allometry of development has long been of interest to developmental physiologists (Gould, 1975; Weder and Schork, 1994; Stern and Emlen, 1999; Singer and Mühlfeld, 2007; Vea and Shingleton, 2021). However, a fundamental challenge that remains unresolved involves reconciling two basic yet conflicting tenets of allometry and development (Burggren, 2020b). Thus, a basic tenet of allometry is that animals of different sizes being compared should be in the same physiological state. Yet, a basic tenet of development is that developing animals are constantly changing physiological state. How then, can allometric analyses simplistically involve both a larva or fetus as well an adult? This is not an insurmountable problem, as there are likely to emerge (or, at least *should* emerge) weighted statistical approaches that take changing physiology into account as allometric analyses are performed (or vice versa).

CHALLENGE: IMPROVING EXPERIMENTAL APPROACHES

Seeing Development as a Continuum Rather Than Discrete Events

Traditionally, developmental physiology has been built upon individual studies carried out at a single or just a few discrete points in development. Yet, I posit that the most robust understanding of "how physiology develops" lies in considering a *continuum* from germ cells to organismal senescence (as encapsulated in the description of the Developmental Physiology Section of Frontiers in Physiology). Any attempted synthesis of developmental physiology gleaned from analysis of multiple physiological studies each conducted at only a single point of development will, at best, be tedious and, at worst, lead to a biased or inaccurate conclusion. By considering a continuum, physiological measurements can more readily be put in context of an organism's entire life cycle. Thus, a specific challenge is to promote experimental protocols that gather data along multiple points of the developmental continuum.

Expanding (and Verifying) our Animal Models

The use of animal models is, of course, a stalwart of developmental physiological investigation. Whether the larvae of zebrafish or *Xenopus*, the chicken embryo, or the mouse or sheep fetus, animal models have been of major benefit to both enhance our basic understanding of the "biology of development" as well as provide information of translational importance to biomedical research. Additionally, invertebrate models including the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* have, as animal models, provided great insight into development. Yet, the challenge is to not become complacent in our use of these animal models. As the Danish physiologist and Nobel Prize winner August Krogh declared, for every physiological question there is the ideal animal to answer it (Krogh, 1929). Initially successful animal models become entrenched in the research community, sometimes resulting in the lack of penetration or marginalization of alternative animal models that could prove to be superior in some respects (Flores Santin and Burggren, submitted).

Experiments Incorporating Multiple Stressor Experiments and Stochasticity

By the nature of our training, physiologists prefer to hold constant all but a single dependent environmental variable of interest (e.g., temperature, pH, oxygen), which is then controlled or allowed to vary. Yet, in the "real world," of course, multiple variables are constantly changing concurrently. Thus, aquatic environments may be hypoxic because they are also warm, while high altitude terrestrial environments may carry have low oxygen and high radiation loads combined with low temperatures and humidities. In recognition of this, many developmental physiologists are beginning to expose developing animals to multiple rather than single changing stressors (**Figure 3**).

What is emerging from "multiple variable experiments" is that different organ systems and their system-specific physiologies have different critical windows for development. A challenge is to generate enhanced understanding of the interaction of development, time and environment through multi-variable experiments (Mueller, 2018).

Riding the Wave of Technological Innovation

Developing animals are almost inevitably small, if not microscopic. Not surprisingly, then, miniaturization has long been a priority in instrumentation for developmental physiology (Burggren and Fritsche, 1995). Several recent technological developments have been aiding the assessment of physiological processes in progressively smaller (and thus earlier) developmental stages. Advances in imaging continues to improve our understanding of especial cardiac physiology of embryos, fetuses and larvae (Aguet et al., 2020; Salman and Yalcin, 2020; Lopez and Larina, 2021). The advent of microfluidics is contributing to our knowledge of embryonic physiology, especially as it pertains to *in vitro* embryo production (Wheeler and Rubessa, 2017; Sonnen and Merten, 2019). Organoids ("organs on a chip") are increasingly being studied either on their own or as components of increasingly complex "organ" systems (Matsui et al., 2021). The study of organoids may yield extraordinary new insights into the assembly of the cells and tissues in early development. These and other emerging techniques typically produce vast amounts of data, often in the form of complex images. Not surprisingly, then, developmental biology, including developmental physiology, has begun to exploit to machine ("deep") learning, which can analyze large data sets without direct human involvement and the associated

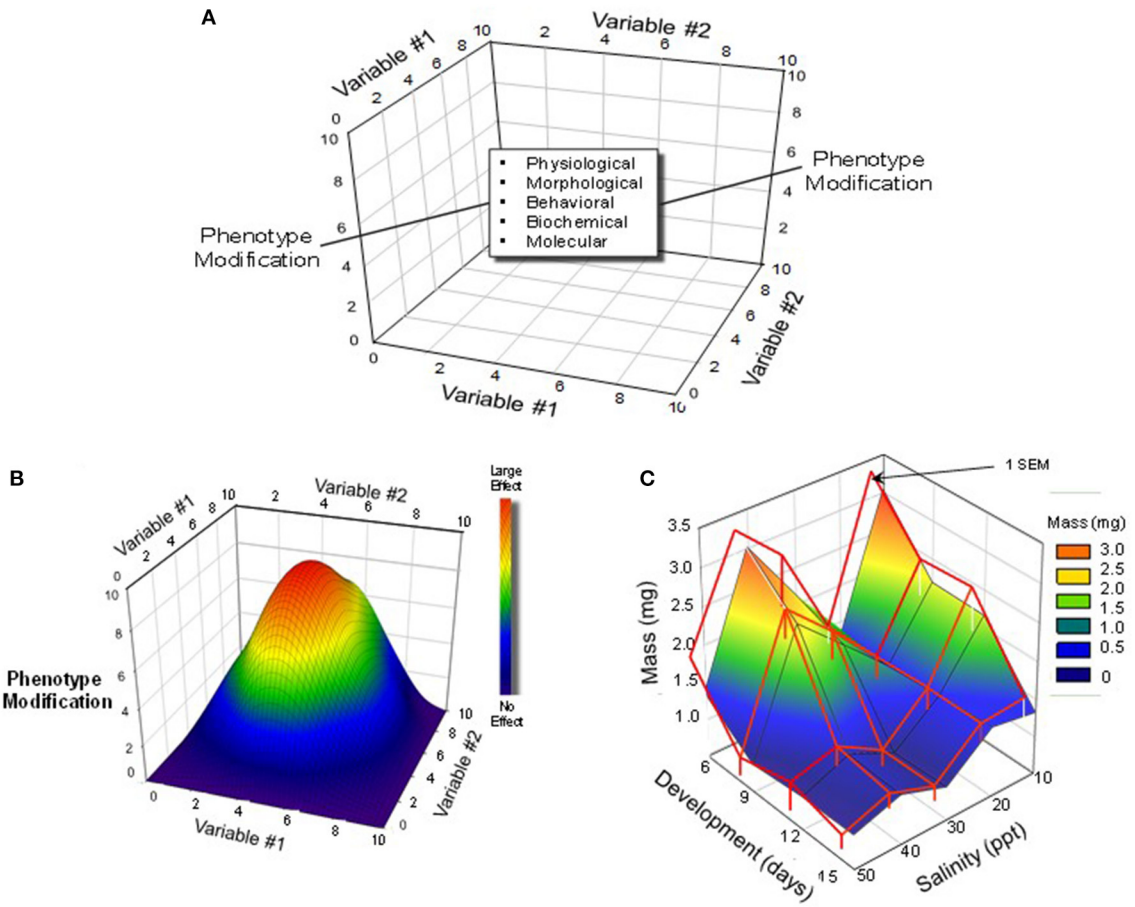

FIGURE 3 | Multi-variable experimental designs and possible outcomes. **(A)** A three-dimensional schematic depiction of a protocol that would employ multiple levels/concentrations/doses of multiple variables. **(B)** A hypothetical outcome on a developing animals phenotype produced by simultaneous variation in two different variables. In this schematic, the largest effect is created by a combination of intermediate levels Variable #1 and Variable #2. Modified from Burggren and Mueller (2015). **(C)** An actual example of interactions between development (Variable #1) and rearing salinity (Variable #2) in the brine shrimp *Artemia franciscana*. Note that there was an unexpected bimodal effect of rearing salinity, with the largest effect appearing early in development. Modified from Mueller et al. (2016).

risk of human-created error (Feltes et al., 2018; Villoutreix, 2021).

There are challenges associated with these and numerous other emerging technological innovations in the engineering processes, which continue to inexorably advance. Yet, the greater challenge may be providing broad access to these technologies and the training required to deploy them.

CONCLUSIONS

In this short perspective on Grand Challenges in Developmental Physiology I have indicated directions in which the field of Developmental Physiology might (not necessarily should) move. Some of my predictions and suggestions will be on target, others less important than predicted, and new unimagined Challenges will emerged. What is crucial is that Developmental Physiology does not become complacent regarding its many achievements, continues to thrive on collaboration with other physiological disciplines, and ensures that its trainees are moving to the next stages of their careers with the skills and enthusiasm that continues to be a hallmark of developmental physiologists.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

ACKNOWLEDGMENTS

I thank the many colleagues and students (and students who have become colleagues) who, through animated discussions over the years, have provided me with most of the ideas outlined in this brief article.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 30 March 2021
doi: 10.3389/fphys.2021.650550

The Physiology of Reproduction – *Quo vadis*?

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The reproductive system in males and females reflects a highly dynamic underlying physiology. Yet our current understanding of this system is still largely based upon relatively simplistic snapshots of individual component cells and tissues. Gamete production as well as gonadal hormone synthesis and its influence are the manifestations of dynamic and redundant informational networks and processes, whose qualitative and quantitative dimensions, especially through development from embryo through puberty and adulthood into ageing, are still largely uncharted. Whilst the recent huge advances in molecular science have helped to describe the components of the reproductive system in ever greater detail, how these interact and function in space and time dimensions is still largely obscure. Recent developments in microfluidics, stem cell biology, and the integration of single-cell transcriptomics with tissue dynamics are offering possible methodological solutions to this issue. Such knowledge is essential if we are to understand not only the normal healthy functioning of this system, but also how and why it is affected in disease or by external impacts such as those from environmental endocrine disruptors, or in ageing. Moreover, operating within a complex network of other physiological systems, its integrational capacity is much more than the generation of male and female gametes and their roles in fertility and infertility; rather, it represents the underpinning support for health and well-being across the lifespan, through pregnancy, puberty, and adulthood, into old age.

Keywords: hormonal networks, parameter variance, pulsatility, endocrine disruptors, uterine peristalsis, systems biology

OPEN ACCESS

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Physiology

Received: *07 January 2021*
Accepted: *11 March 2021*
Published: *30 March 2021*

Citation:
Ivell R and Anand-Ivell R (2021) The Physiology of Reproduction – Quo vadis? Front. Physiol. 12:650550. doi: 10.3389/fphys.2021.650550

INTRODUCTION

Physiology has been defined as the "*science of health*" as well as the "*science of life*." For reproductive physiology, this is not unproblematic since much of what we consider as reproduction is not essential to either. At an individual level, disorders of the reproductive system such as infertility are generally not life-threatening, nor lead to major bodily dysfunction. Whilst some might consider that infertility is not a disease, at the population level, any disruption of reproductive function can lead to loss of a population or even a species. And at the individual level, such malfunction can lead to considerable personal grief. Accordingly, in the competitive jungle of research funding, reproduction does not compete well with cancer, diabetes, or neurodegenerative illness, even though fertility problems may impact a larger number of people, especially as child-bearing is being delayed until the mid to late 30s or beyond. Our field is further disadvantaged by a minimal

engagement from the pharmaceutical industry, for whom the reproductive sector is a litigious minefield because of the subtle risks associated with exposures of an unborn child. Consequently, most drugs used today still have their roots in substances discovered many decades ago, and which if invented today would probably find it difficult and expensive to receive approval. Finally, the relative success of Assisted Reproductive Technology (ART) has lulled society into thinking that infertility issues are largely resolved.

A consequence of such attitudes is that research into fundamental reproductive physiology has not progressed as much as in other fields and owes its advances often to translation from different sectors of physiology. Moreover, when we assess the articles being submitted to our specialist *Frontiers* section on "Reproduction," by far the majority represent incremental improvements to existing clinical procedures and applications, and relatively few contributions to innovative basic science.

In this essay, we should like to draw attention to why Reproductive Physiology merits more fundamental research – in particular, into those aspects which differ from other areas of Physiology. This is intended neither as a comprehensive review of recent breakthroughs and successes, nor as a detailed list of future research targets. Rather, we want to raise ideas to stimulate innovative approaches to understanding the field as well as its applications into a broad array of translational targets.

THE DYNAMIC PHYSIOLOGY

Most areas of physiology are governed by a relatively static image of an adult system, whose rules and boundaries are controlled by homeostasis. There is a normal (healthy) state where components have fixed relationships to one another, which may become altered in a disease condition. In contrast, the reproductive system is never static. In the adult female, reproductive status is ruled by the dynamic depletion of the follicle reserve, from birth to old age, superimposed upon which is the menstrual cycle, or its equivalent, regulating not only the final preparation of the egg for fertilisation and eventual pregnancy, but also the organs of the reproductive system such as the uterus and mammary glands, as well as other systems in the body such as the bone and skeletal system, the osmotic balance, the immune system, and especially the brain and behaviour. In the male, there may be no monthly cyclicity, nor limited gamete reserve, but the system is still very dynamic, with continued spermatogenesis from stem cells throughout adult life. With the possible exceptions of intestinal epithelial sloughing and haematopoiesis, no other organ system is adapted to producing continually many millions of cells a day whose function is external to the organism, and which must be capable of generating perfectly functional individual cells (spermatozoa) for many decades.

The parameters governing both male and female reproductive systems – even in the adult – are therefore never static, but to be seen always as snapshots of very dynamic processes, the dimensions of which can at best only be approximated, much as a photograph can never display the living reality of the subjects.

However, the reproductive system also reciprocally dictates the health of the organism, not only in adulthood, but especially during prenatal development, puberty, and in senescence, adding further dimensions to the dynamic interplay of the cells and organs involved. Events occurring early in development can have big effects at later stages, as in the DOHaD (Developmental Origins of Health and Disease) concept. It is our lack of understanding of the dynamic processes involved and their regulation which may account for the fact that many of the commonest diseases affecting us are those involving the reproductive system and its dynamic development (polycystic ovarian syndrome, endometriosis, fibroids, premature ovarian insufficiency, male and female infertility, breast, endometrial, and ovarian cancers, benign prostatic hyperplasia, testis and prostate cancers, and hypoandrogenemia).

An aspect of this, which is not often appreciated, is the large variation in clinical and biological parameters in the human population associated with reproduction, which we consider "normal" and non-pathological. Part of this may reflect the point raised above that, since disruption of the reproductive system is rarely life-threatening, we are more tolerant of variation *per se*. For the male, for example, the WHO latest acceptable reference range for sperm concentration is 15–259 million per ml with progressive motility of 32–75% and for normal morphology is only > 4% (Cooper et al., 2010; WHO, 2010)! We have shown that the normal range for circulating insulin-like peptide 3 (INSL3), a measure of Leydig cell functional capacity (i.e., total number of Leydig cells in the testes compounded with their differentiation status) is 0.39–1.87 ng/mL (5%, 95% confidence intervals) in healthy young men (Anand-Ivell et al., 2021). This is a large variation, compared to other, comparable non-episodic health parameters, such as thyroxine (5.5–12.5 μg/dL) or haemoglobin (14.0–17.5 g/dL). For women, there is a similar large range for the age at natural menopause (40–58 years) or circulating AMH (6.4–67.9 pmol/L in reproductive age women), both a reflection of egg reserve. It is important to note that these are quasi-constitutive parameters not subject to acute feedback control, for example, by the hormones of the hypothalamo-pituitary-gonadal (HPG) axis. Yet we understand very little about the causative factors involved in this large variation in reproductive parameters, except that they probably reflect events early in development either before birth or during puberty. Nor do we fully understand the roles played by ethnicity and genetics in establishing these normal ranges.

Such reproductive parameters probably depend upon stem cell proliferation and differentiation, the important features being the relative timing of these two processes and how that timing is controlled. There are similarly dynamic mechanisms affecting cell death (apoptosis) in these lineages. Thus, reproductive physiology must be seen as a complex interaction between multiple highly dynamic processes. Yet most of our knowledge and understanding is based upon sporadic snapshots of mostly adult scenarios.

A further exacerbating factor is that we know most about very few species, such as the human or the mouse, and then try to extrapolate and adapt this knowledge to other animals. Whilst this may be reasonable for some species, such as ruminants, or primates, it can become problematic for exotic and endangered species, or lower vertebrates, whose physiology is often nebulous at best. There is therefore a need for elaborating more and alternative animal models to help delineate these complex processes.

INFORMATION THEORY 101

Information theory, originally derived from telecommunication to describe information transfer and its efficiency, is highly pertinent to any understanding of how living systems work, yet has surprisingly found little mention in basic biology texts. The flow of information – its production and its reception – is the key difference between living and non-living, between life and death, and between health and disease (Yockey, 1992). Because the reproductive system is, by definition, very ancient and essential for the survival of the species, we see a wide range of informational systems being used, both paracrine and endocrine. The HPG axis represents a classic example for information transfer at a distance. Not only are the gonadotropin hormones that are produced by the pituitary gland, complex information carriers whereby multiple peptide and carbohydrate epitopes within the same molecule all need to be correctly "seen" by their cognate receptors, thus minimising misinformation, but the system additionally employs both pulsatility (digital encoding) and negative feedback to ensure correct and adequate information transfer.

Both gonadal steroids and certain peptide hormones (e.g., inhibin B) feed back to the hypothalamus and pituitary to regulate the output signal. Whilst we now understand much of the qualitative and anatomical detail, surprisingly, we know only very little about the quantitative aspects involved here. How do those cells receiving the feedback quantitatively measure the amounts of gonadal steroids, inhibin B, or perhaps other molecules feeding back information; how are these feedback signals quantitatively integrated to generate not just a yes/no secretion response but an appropriate quantity of a particular hormone by the hypothalamus and/or pituitary? Regarding pulsatility, we still know very little about the detailed mechanisms involved. It is 40 years since the pioneering study by Wildt et al. (1981) which showed for macaques that GnRH pulse frequency determined the quantities of gonadotropins released and whether preferentially FSH or LH or both. Although Johannes Veldhuis and colleagues have elaborated examples of the changes that occur in gonadotropin pulsatility with development, sickness, or ageing (e.g., Veldhuis et al., 1994; Liu et al., 2005), little of this knowledge has yet been practically translated into clinical application. We still rely upon single measurements of peripheral hormones, hopefully at a similar time of day, or at best a GnRH challenge test. There is obviously a major technical obstacle here: how can we measure and interpret pulsatility in a routine clinical context? Only few studies have tried to approach this issue (e.g., Henley et al., 2009; Liang et al., 2019; Poudineh et al., 2020). Given our advances in other areas of technology, it is surprising that we seem to be no nearer to harnessing the informational content in pulsatility, especially when we consider how many different hormone systems appear to make use of it and how important it might be for the understanding of health and disease. We could learn from findings from other pulsatile hormones, such as parathyroid hormone, where loss of appropriate pulsatility (rather than its concentration) is a biomarker of osteoporosis (Chiavistelli et al., 2015). Conversely, administration of hormones or drugs in a pulsatile fashion is still very limited. Rather, hormones, such as GnRH analogues, may be administered as a single, high-concentration bolus specifically and chronically to desensitise and hence inactivate their receptors and the relevant feedback loop. Even for oxytocin, another well-characterised pulsatile hormone, it is still advocated at labour to deliver it as a continuous rather than a pulsed infusion (e.g., Tribe et al., 2012); even though it requires higher amounts of the hormone and can lead to oxytocin receptor desensitisation, including possibly within the foetal brain and leading to a risk of autism (e.g., Weisman et al., 2015). A greater understanding of how hormones are encoded and decoded in pulsatile systems could open up many new opportunities for drug-based therapies, besides expanding our understanding of disease.

NETWORKS, NOISE, AND VARIANCE

Historically, the great successes achieved in combatting disease with single drug replacement strategies, such as the application of insulin in diabetes, or thyroxine in hypothyroidism, have created a dogma that most physiological systems can be similarly "fixed" by activation of a simple pathway; conversely, that if a gene product is important, its knockout or removal must show a clear phenotype. The reality that is especially being highlighted by the "big data" approaches of GWAS studies, or global NGS-sequencing, is that many genes may be involved in a particular phenotypic pathway, none of which individually may deserve the appellation of being "causal" or "essential." Rather genes and their products are linked together into complex and dynamic networks whose functionality can tolerate a degree of redundancy, but which are nevertheless holistically essential for the appropriate function of a cell or tissue.

A good example is provided by the dialogue between the blastocyst and the endometrial lining of the uterus at the time of implantation. We now understand that mutual and multiple signals from both the blastocyst as well as from the receptive endometrium interact in a largely temporally sequential fashion, subsequent steps being "mostly" dependent on successful "hand-shaking" of preceding steps. The term "mostly" is highlighted because this system allows a modest redundancy, both in molecular and in temporal terms. Only when the negotiation is satisfactorily finalised can the blastocyst be considered as implanted and placentation can proceed. The negotiation involves quality checks: on the embryo to determine

whether it has the potential to develop into a healthy offspring and on the mother to test whether she will be able to take this baby to term. If the quality checks fail, then there is miscarriage, preferring to reserve energy for a new pregnancy rather than risking failure at a later stage (e.g., Macklon and Brosens, 2014; Valdes et al., 2017; Craciunas et al., 2019; Ewington et al., 2019).

In such a system, it is difficult to identify single key molecules or biomarkers essential to successful early pregnancy; in general, the dynamics of the negotiation process are very hard to measure. This is even more difficult in species where the apposition time of the embryo to the uterine lining is much longer than in the human. In analogy to the "quiet embryo" hypothesis (Baumann et al., 2007), it is likely that when the negotiation is rapid and signalling within this network is relatively quiet, it is a sign of a healthy implantation process; in contrast when it is protracted or "noisy" with much information flow, this could be an indication that the process is problematic with a high risk of failure. But how can we assess such a dynamic process? Importantly, how can we manipulate this process, and should we? By overriding this quality control dialogue, there is a risk that we might be retaining an offspring which otherwise might have been subject to natural selection. But what if the quality control process itself is faulty? One consequence of this hypothesis might be that molecular parameters will show a higher level of "noise," or variance, with a problem outcome, though may not show alterations in mean parameter levels.

Consider, for example, the situation during ART when multiple healthy-looking embryos are transferred and only one succeeds to implant. Was the decision here a quantitative one, or qualitative, or a dynamic combination of these?

A related example concerns the extent to which informational processes may involve stochastic mechanisms. During the early cell divisions of the fertilised egg, the resulting morula comprises more-or-less identical blastomeres, each of which can, if separated, give rise to an independent individual. Early blastomeres are totipotent. There are few geographical clues until the blastocyst stage, except for those resulting from the point of sperm entry, and any unequal distribution of the original egg cytoplasm and its contained gene products. Only later, with the formation of the blastocoel, inner and outer layers, and the initial trophoblast, can clear polarity be established, and cell lineages become determined. However, attempts to use individual blastomeres as diagnostic tools to deliver prognosis on the resulting embryo have generally not succeeded, largely due to the very heterogeneous nature of blastomere gene expression profiles. One explanation for this could be that initially gene expression in the early morula is mostly a stochastic process rather than being deterministic. The blastomere genomes are mostly not epigenetically determined once the zygote is formed and will anyway undergo an erasure phase in the blastocyst. The stochastic activation of genes would have two consequences: first, it could establish a random informational (chaos) network which could then progressively by feedback fixate upon certain pathways and relationships and second, it could provide an active mechanism for proof-reading the genome. Both mechanisms would have definite advantages for the survival of the offspring. From a pragmatic perspective, such a stochastic mechanism would also explain why there may be an upper limit to the success rate of *in vitro* fertilisation (IVF), since any such proof-reading step implies the necessary loss of inadequate zygotes. But such a mechanism would also explain why IVF succeeded in the first place, since it would intrinsically be able to rectify any potential damage caused by the unusual IVF environment. It will be very interesting to find a way to test such a paradigm and the resulting informational networks and thus redefine the impact of *in vivo* and *in vitro* culture conditions, especially in the context of the infertile patient or the endangered species.

DEVELOPMENT: BLURRING THE BOUNDARIES

The relatively recent relaxation of socio-political boundaries has introduced the concept of gender fluidity. Until only a few years ago, scientists were seeking and finding molecular mechanisms which by mutual inhibition would ensure the polarity of the sexes and thus the prerequisite for reproduction and the continuation of the species. Classic examples of this are the expression of the SRY gene on the Y chromosome (Koopman et al., 1990), or the reciprocal inhibition of FGF9 and Wnt4 (Kim and Capel, 2006) during early gonad development. The demonstration of such examples unconsciously suggests that subsequent unclear sex is likely due to aberrations of development, with research then focussed on singular defects of an otherwise perfect system. However, we now realise that gender physiology is not as polarised as we once imagined; it appears more plastic, whereby dynamic and complex informational networks, as discussed above, can be influenced in more than one direction. This would allow a degree of redundancy in the system and a possible blurring of the boundaries.

To illustrate the issue let us consider the foetal gonadal hormones. We are used to considering that phenotypic androgenisation is brought about by testosterone produced by the foetal testes within the so-called "masculinisation window" (Welsh et al., 2014). Less well known is the fact that female foetuses produce almost half the concentration of testosterone as male foetuses (Ivell et al., 2017), mostly from the foetal adrenal gland, and certainly more than sufficient to influence androgen-sensitive systems. Not only that, but in multiparous species such as rodents or pigs, gonadal hormones like testosterone or the peptide INSL3 can transfer between male and female foetuses *in utero* (Vernunft et al., 2016), without having any apparent effect on the female phenotype. It is argued that in the female foetus, the receptors for these hormones are suppressed and therefore unresponsive, but then we need to ask why the androgen receptor in mammals has been evolutionarily segregated to the X chromosome, where there is a greater likelihood of it being expressed in females or recessively mutated in males. Could the solution be that whilst we know a lot about the qualitative response of cells to hormones, we still know very little about the quantitative response: how much hormone is

sufficient to elicit an appropriate response *in vivo*? Evidently, we still understand far too little about the qualitative and quantitative complexity of the informational networks governing the phenotypic expression of gender.

We also need to consider why the reproductive system has conserved the components of the HPG axis across the whole of chordate evolution, and yet when it comes to sex determination appears to employ a wide diversity of genetic systems in mammals, birds, and reptiles. For a physiological system which is absolutely essential for the survival of the species, this is indeed surprising, and suggests that there is much that we still do not comprehend.

ENDOCRINE DISRUPTORS

Reduced reproductive performance was one of the first physiological systems to be recognised as being negatively influenced by endocrine disrupting chemicals (EDCs) in the environment (Gore et al., 2015). These pervasive manmade substances (pesticides, plasticisers, fire retardants, microplastics, etc.) are now known to influence most organ systems of the body, often at very low exposures and concentrations, and ambient levels are increasing. Importantly, they appear not to conform to conventional toxicological paradigms, but can show non-monotonic dose–responsiveness even down to very low concentration. Moreover, because of epigenetic changes, they may also impact later life periods and subsequent generations. Among their key characteristics (La Merrill et al., 2020), they appear to function by interfering with diverse endocrine components (hormones, receptors, transporters, enzymes, etc.). However, in the light of what has been discussed earlier, they may function additionally by subtly modulating informational networks. One consequence of this is that effects may be due less to absolute changes in endocrine parameters and more to increased noise or variance in such informational systems. Such an effect was suggested in assessing the effect of phthalate exposure on the risk of cryptorchidism or hypospadias in male human foetuses (Anand-Ivell et al., 2018).

The biggest problem to be faced with regard to EDCs is that they often appear to be insensitive to conventional regulation applying toxicological paradigms. Regulatory agencies require more and novel approaches if they are to be able to keep up with the many millions of tons of such substances released into the environment every year. While some *in vitro* or *in silico* methods are showing considerable promise, these work only by assuming that EDCs are mimicking natural hormones and their interactions with receptors. Yet, as we have shown above, EDCs appear to illustrate a range of diverse endocrine properties, and moreover may function within the context of dynamic and redundant networks, so that such relatively simplistic approaches may fail to recognise a majority of potential EDC compounds.

A further complication in the interaction between EDCs and reproductive physiology is that we now recognise that also many pharmaceutical compounds are behaving similarly. Initially, it was discovered that the contraceptive component ethinyl oestradiol was entering natural water systems relatively unscathed and having potent effects to feminise male fish (Jobling et al., 2006). Therefore, precisely those properties which make a pharmaceutical resistant to degradation in the body are also those that allow it to persist in the environment once excreted (Heberer, 2002). Thus, our marine and freshwater environments are having to cope with increasing amounts of common pharmaceuticals, including contraceptive agents, analgesics, hypertensive drugs, etc. Their impact on food chains is still largely uncharted.

Also of concern is that we are discovering that many pharmaceuticals, previously considered relatively harmless, are now seen as impacting on foetal development. The analgesics, paracetamol (acetaminophen) and ibuprofen, have been shown in animal models as well as in human epidemiological studies to affect the development of the foetal gonads following exposures during pregnancy (Kristensen et al., 2016; Kilcoyne and Mitchell, 2019). The issue here is that previous regulatory strictures regarding testing evidently did not take account of the subtle mode of action of EDCs, particularly on the reproductive system or during pregnancy. Research is still at a very early stage here, and it is likely that the future will reveal that several common or over-the-counter pharmaceuticals may be found to have EDC-like effects. Particularly concerning in this regard is also the massively increased prescription of psychoactive drugs, such as SSRIs, to women of reproductive age, and to adolescents going through puberty (Fenger-Grøn et al., 2011; Grzeskowiak et al., 2012). These are categories which we know are likely to be very sensitive to the effects of potential endocrine-acting compounds.

AGE AND REPRODUCTION

It is the purpose of any species, once it has reached sexual maturity, to reproduce and secure the continuation of the gene pool. Humans are different. The attainment of sexual maturity at the end of puberty is separated by several years both from parenthood itself, as well as from the behavioural maturation that is required for a successful family. There are three sequelae to this. First, women experience a succession of futile physiological preparations for pregnancy without ever becoming pregnant. Evolution has not prepared women for this. Every month cyclic hormones activate stem cells to begin preparing the endometrium or breast tissue for an eventual pregnancy without this event actually occurring, and a default programme is then invoked to reset the calendar. Besides the accompanying menstruation and/or mental disruption this provokes, it has been also suggested that this activation/reset system is one factor predisposing women to reproductive tissue cancer (Olsson and Olsson, 2020). Second, contraceptive systems have been developed to provide planning for reproduction to coincide with an optimal economic environment. The commonest contraceptives for women still rely on manipulating the cyclic hormones following a paradigm which is now more than 60 years old. Whilst choice and alternatives also for men are needed, few new concepts have found acceptance. Third, socio-economic pressures have pushed the age at which families are started into the mid-30s or

older. Fertility in women declines rapidly from the early 20s onwards, with mean TTP (time-to-pregnancy with unprotected intercourse) shifting from about 3 months at age 20–25, to 6 months or longer at 30–35 years, and >24 months by the age of 41. Whilst modern ART procedures have done much to alleviate such infertility issues, and even the freezing of youthful ovarian tissue or oocytes has progressed to mainstream, there are inevitably consequences of such alterations of biology for which evolution has not prepared us. Maternal and paternal age is known to be not only associated with increased genetic risk for the offspring, but also subtle epigenetic effects which may only become apparent later in life (Aiken and Ozanne, 2014; Preston et al., 2018).

There is still much we do not understand about the physiological impacts of this shift in family planning. But increased life expectancy has also thrown emphasis on the endocrine roles played by the gonads in older age. The gonads and reproductive system not only serve the production of offspring, but their hormones contribute majorly to health and overall well-being. The exhaustion of the ovarian follicle reserve in women at mean age 51 years signals the menopause, though this is not an abrupt transition but may progress over several years as the resident follicles slowly lose their capacity to produce hormones. Until recently, loss of follicles was considered synonymous with loss of the sex steroids oestradiol and progesterone, and to date hormone replacement therapy (HRT) has focussed only on these two molecules. However, it is becoming clear that the follicles also produce other hormones, such as the gonadal peptides INSL3 or relaxin, which are also lost to the circulation at the menopause. Recent studies in mice and humans have shown that loss of INSL3 expression or of its specific receptor leads to osteopenia or osteoporosis (Ferlin et al., 2008), and relaxin has markedly positive effects on cardiovascular physiology and fibrosis (Leo et al., 2019), indicating that future HRT concepts might need to consider replacing additional hormones.

In men, similarly, the gonads also produce peptide hormones, such as INSL3, in addition to steroids like testosterone. Importantly, these also decline significantly with increasing age. Whereas testosterone declines at between 5 and 7% per decade, INSL3 declines at almost 15% per decade, since the latter, being quasi-constitutive, is not acutely compensated by increasing gonadotropins (Anand-Ivell et al., 2006) and is a true reflection of the age-dependent decline in Leydig cell functional capacity. Besides a possible role for INSL3 in osteoporosis in men, this decline in Leydig cell function adds to the growing debate about whether or not to supplement older men with testosterone. At present, HRT for men is mostly restricted to those exhibiting specific late-onset hypoandrogenemia (LOH), which affects only about 2% of elderly men and is characterised by specific androgen-deficiency symptoms (Basaria, 2013; Pye et al., 2014). Further research is evidently required to explore this age-dependent endocrine decline in men which accompanies increased frailty, cognitive decline, and increased incidence of metabolic syndrome. A new kind of HRT concept for men, with broad application, could go a long way to alleviating many of the debilitating ailments affecting men as they get older. In both men and women, the gonads are not just meant to produce gametes, but contribute to overall well-being and general health.

BIG DATA

Recent years have witnessed a revolution in our ability to gain information about genes and their expression, with technologies such as GWAS analysis, MS-proteomics, NGS RNA-sequencing, as well as microarrays, allowing for the first time a global and comprehensive approach to measuring molecular events in cells, tissues, and organisms. In turn, this has led to many new statistical methods to distil out key events and pathways from these large datasets. Whilst no doubt providing much new information about physiology, these approaches still only achieve a molecular snapshot of what is going on. As alluded to the above, part of the difficulty is that we are still treating the objects of our study using simplistic paradigms and hypotheses, which ignore the dynamic nature, the complexity, and the redundancy, of what is driving physiology, particularly in reproductive tissues and organs. Many recent "big data" studies have not succeeded in, for example, identifying key biomarker(s) for healthy endometrium, or healthy follicles, or fertile sperm. Rather they illustrate redundancy, or place a probability on a pathway, with few if any studies coming up with equivalent and comparable results for a particular target. The problem is not the methodology, but the over-simplistic hypotheses that we apply. Evolution has had many millions of years to perfect informational systems which are robust, redundant, highly dynamic, and flexible. Such systems are rarely binary, often subtle, reflecting small changes in sensitivity, and can involve shifts in synchrony as much as quantitative shifts of expression. We need to invest research into a better understanding of such systems, using computer modelling and systems approaches in addition to discovery in order to extend our frontiers.

PHYSIOLOGY IS MORE THAN MOLECULES, E.G., UTERINE PERISTALSIS

Molecular analysis has dominated recent decades of research in reproduction and has the advantage of providing hard, quantifiable data. It maps the molecular skeleton upon which physiological forces can act. But it does not answer the question of how things work. This deficit may explain in part why reproduction as a clinical discipline is still reliant on many surgical procedures as treatments, such as hysterectomy, prostatectomy, or caesarean section.

A good example is provided by the topic of uterine peristalsis. Like the gut, the uterus is also enclosed within sequential layers of longitudinal and circular smooth muscle and is subject to directed peristaltic waves of contraction. At the most receptive time of the menstrual or oestrous cycle, these contractions are mostly in the direction cervix to fundus (i.e., from bottom to top) coinciding with the opening of the valve or isthmus between the uterus and the oviduct on the ovarian side producing a

fertile oocyte. This is the optimal time for intercourse, and a bolus of ejaculated sperm will pass the cervix and be transported by a peristaltic wave to the open isthmus and to the oviduct for fertilisation (Kunz and Leyendecker, 2002; Kuijsters et al., 2017). Pituitary oxytocin and uterine oxytocin receptors may also be involved in this contractility (Kunz et al., 2007). Later in the cycle, the directionality of uterine peristalsis reverses; the isthmus to the oviduct is closed and contractions serve to flush out menstrual fluid through the cervix. It is believed that the condition, endometriosis, whereby endometrial tissue grows ectopically on peritoneal membranes or on the ovarian surface, may be initiated by a dysperistalsis causing menstrual debris including endometrial stem cells to be flushed in the wrong direction through the oviduct and into the peritoneal cavity (Child and Tan, 2001). Similarly, uterosalpingography, and more recently cine-MRI, have shown that in infertile women, instead of normal cervix-to-fundal peristalsis at ovulation, the waves are reversed or irregular, thereby preventing sperm from fertilising the egg. Moreover, it is believed that the presence of fibroids is also a key factor in disrupting uterine peristalsis (Pier and Bates, 2015). Furthermore, in some species, producing multiple embryos, regular, standing peristaltic waves may be responsible for spacing of the embryos within the uterus.

We know very little about how uterine peristalsis is controlled. In analogy to the gut, it appears that there may be resident Cajal-like cells, or telocytes, in the uterus, which acting as pacemakers could coordinate myometrial contractility, in conjunction with the autonomic nervous system (Hutchings et al., 2009; Banciu et al., 2016). There does not appear to be any steroid-induced geographic expression of oxytocin or other receptors. Considering how important the observations described above may be in relation to some of the major diseases affecting human reproduction, endometriosis, fibroids, and/or infertility, as well as conditions such as premature labour, it is perhaps surprising that so little is known about this classical aspect of physiology. But then this is not an aspect which can be easily assessed using modern molecular techniques. New developments in cine-MRI are making the assessment of uterine peristalsis more accessible, though this is still some way from understanding causality or identifying possible treatments.

At a cellular level, it has recently been shown for the rat uterus, by combining electrophysiology using microelectrode arrays and microanatomy, that contractility in late gestation appears to be initiated by a "pacemaker" domain anatomically defined as a specialist region of the myometrium adjacent to placental tissue (Lutton et al., 2018). How such domains function and whether the concept applies to other times besides late gestation, or in other species, remains to be clarified.

BIODIVERSITY, BEHAVIOUR, AND THE BIGGER PICTURE

The loss of biodiversity and loss of endangered species are also aspects touching the physiology of reproduction. This may be direct in that the reproductive system of a particular species is suffering dysfunction through exposure to environmental chemicals, or genetic inbreeding and an environment to which the species is not well adapted. Or it may be indirect because the behavioural context for appropriate reproductive physiology is dysfunctional. For example, it has been shown that EDC exposure can upset mating behaviour in rats without having any other obvious molecular or physiological consequences. Without going into detail, it is important to note that only when an integrated physiological approach is taken, which considers all aspects pertaining to reproduction (genetics, behaviour, ecology, endocrinology, and reproductive physiology) will it be possible to understand and thus develop solutions to the current biodiversity problem. While modern approaches to develop ART procedures suitable for endangered species, such as rhinoceros, are essential and laudable, only when these can be integrated into the larger physiological and environmental context of habitat renewal and behavioural monitoring, can any success be expected. Thus, the importance of taking a physiological approach in reproductive science is that it – by definition – is integrative and inclusive of a wide range of methodologies, rather than being constricted by a discipline-based focus on the molecular or the genetic.

LIGHT ON THE HORIZON—NEW METHODS AND APPROACHES

As outlined above, the static cell-molecule approach needs to transmute into a more dynamic and integrated systems methodology. The progression of stem cell biology and the development of lineage into more complex *in vitro* models using organoids are already providing important dynamic information about cell–cell interactions. Similarly, the evolution of microfluidics systems allowing informational integration between different organ components is beginning to illuminate the reciprocal dynamics of the oestrous cycle (Xiao et al., 2017). Combining multiple single-cell transcriptomics across tissue sections is highlighting the dynamic heterogeneity of the endometrium and the placental–maternal interface, besides for the first time functionally identifying the roles of individual cells in their natural context (Vento-Tormo et al., 2018; Garcia-Alonso et al., 2021). This new methodology, like the myometrial electrophysiology mentioned earlier, has in common that instead of homogenising tissue expression data, as in the past, it is celebrating the heterogeneity of single cells and their functionality, and combined with a systems biology computational approach, is allowing us for the first time to appreciate the spatial and temporal dynamics essential to reproductive function.

On a larger scale, mid-gestational foetal-maternal development has always represented a relatively inaccessible "black box," whose disruption is considered critical in terms of maternal and foetal pathology (pre-term labour, preeclampsia, gestational diabetes, miscarriage, foetal organogenesis, and DOHaD). Access to placental, foetal, and amniotic samples for analysis, and also non-invasive methods are very limited. However, the recent breakthrough development of an artificial placenta and/or artificial womb is providing hope not only for the survival of ever-earlier pre-term infants, but also in terms of

our understanding of the dynamic foetal–maternal interchange (De Bie et al., 2020).

CONCLUDING REMARKS

This essay makes no attempt to be comprehensive or present a balanced summary of current research into reproductive science. Numerous excellent recent clinical and molecular advances have been ignored. Rather it attempts to show how our current focus on molecular detail may be obscuring our understanding of the mechanisms and processes which govern the functioning of our reproductive organs and tissues. These are highly dynamic and require a different kind of approach which is integrative in both space and time. Such new approaches will also probably require different statistical and computational methodologies, besides new techniques and new models. We have learnt to dissect and take apart the system into its component molecules, now it is time to put this knowledge back together again and understand how the complete system works.

AUTHOR CONTRIBUTIONS

RI and RA-I together conceived, authored, and reviewed the manuscript. Both authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

SPECIALTY GRAND CHALLENGE
published: 20 May 2021
doi: 10.3389/fphys.2021.684745

Frontiers in Chronobiology: Endogenous Clocks at the Core of Signaling Pathways in Physiology

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Keywords: endogenous rhythmicity, canonical and non-canonical circadian clocks, photic entrainment, non-circadian clocks, COVID-19

Chronobiology is a relatively young and fast evolving research field, which aims at understanding the origin, the mechanisms and the prerogatives of endogenous biological clocks.

The Chronobiology section of Frontiers in Physiology provides an interdisciplinary forum for the publication of research covering all aspects of the field, including molecular clock circuitry, clock evolution, animal models, physiology, translational studies, and chronotherapy. Over the past few decades, chronobiology has moved from occupying a specialist niche within physiology research, to influencing every aspect at all levels of the discipline. In 2017, the Nobel Prize in Physiology or Medicine was awarded to Jeffrey C. Hall, Michael Rosbash and Michael W. Young, three chronobiologists and drosophilists, "for their discoveries of molecular mechanisms that control circadian rhythms." The field went on to receive considerably more interest and attention, and the efforts of those researchers who had been working on chronophysiology and its medical implications and applications were also rewarded as chronobiology entered its true translational era (Cederroth et al., 2019). This has been characterized by a flourishing of relevant, novel clinical observations (to name one, the fact that the outcomes of certain types of cardiac surgery are heavily dependent on time of day; Montaigne et al., 2018), by the evolution and the definition of an almost entirely novel chronobiology vocabulary and, most interestingly, by experiments and observations that constantly challenge the few true dogmas of this relatively young science. The definition of clock cells themselves has changed, moving away from the idea that there are cells with specific features that qualify them as oscillators to a model where the clock or oscillator results from the interaction of distinct physiological players (circadian networks) (Mizrak et al., 2012). Similarly, it has become evident that brain structures other than the suprachiasmatic nuclei (SCN) clock neurons [for example astrocytes within the SCN itself (Hastings et al., 2019), the habenula (Baño-Otálora and Piggins, 2017) and the blood brain barrier (Cuddapah et al., 2019)] exhibit clock properties or produce oscillations that modulate SCN outputs in many different ways. Thus rhythmicity, both circadian and over other time scales (for example seasonal, lunar and tidal) is transforming into an ever more complex, versatile and interesting natural phenomenon. The influence of chronobiology on society at large has also been profound, resulting in campaigns, for example, to modify urban lighting, to amend school times and to abolish daylight saving time (Roenneberg et al., 2019). Amongst these developments, I will now focus on a few that fascinate me and, hopefully, will stimulate you.

IN SEARCH OF PRIMORDIAL AND NON-CANONICAL CLOCKS

Over the past 15 years or so, evidence has accumulated that non-canonical circadian clocks–i.e., clocks which are not based on the transcription/translation feedback loop (TTFL) that characterizes the molecular timing mechanisms of almost all organisms investigated so far–also exist. Further,

OPEN ACCESS

Edited and reviewed by:
George E. Billman, The Ohio State University, United States

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Specialty section:
This article was submitted to Chronobiology, a section of the journal Frontiers in Physiology

Received: *23 March 2021*
Accepted: *14 April 2021*
Published: *20 May 2021*

Citation:
Costa R (2021) Frontiers in Chronobiology: Endogenous Clocks at the Core of Signaling Pathways in Physiology. Front. Physiol. 12:684745. doi: 10.3389/fphys.2021.684745

they seem to play a significant role in orchestrating the temporal expression of portions of the genome in several organisms. For example, the cyanobacterium *Synecochoccus elongatus* exhibits a circadian biochemical oscillation involving three clock proteins (KAI A, KAI B, and KAI C), generating a post-transcriptional phosphorylation loop, which occurs *in vivo* and can be reproduced *in vitro*, in the absence of transcription and translation (Nakajima et al., 2005). More recently, TTFL-independent oxidation-reduction circadian cycles of peroxiredoxins (i.e., highly conserved antioxidant proteins involved in the control of peroxide levels) have been described in bacteria, archaea, fungi, plants and animals (O'Neill and Reddy, 2011; O'Neill et al., 2011; Edgar et al., 2012). Both KAI B and peroxiredoxins belong to the superfamily of thioredoxins and they may represent conserved relics of the primordial clock of the last common ancestor of prokaryotes and eukariotes. While progress has been made in understanding the molecular mechanisms driving these oscillations, their origin and most of their features remain obscure. There are also indications that a non-canonical clock controls the expression of a significant set of genes, proteins and protein modifications in mammalian cells and tissues cultivated *ex vivo* (Ray et al., 2020). In further detail, cultured (i.e., not under the influence of the SCN) skin fibroblasts and liver slices defective for BMAL1, a transcription factor which is essential for the TTFL-based circadian clock, both exhibit 24-h oscillations of portions of their transcriptome, proteome, and phosphoproteome. The authors propose that this insofar ignored piece of clockwork could result from the interplay of a novel set of transcription factors and non-transcriptionally regulated peroxiredoxin-like redox oscillations (Ray et al., 2020). Nonetheless, the findings remain unexpected and concerns have been recently raised in relation to their consistency, validity and significance (Abruzzi et al., 2021; Ness-Cohn et al., 2021). Finally, the examination and interpretation of available data on the role of circadian and non-canonical clocks in embryonic development suggests that cell division, metabolism and epigenetic modifications become temporally organized before the emergence of a functional TTFL clock (Bedont et al., 2020). Thus a non-canonical, somewhat primordial clock would regulate development throughout cell stem progression toward pluripotency. The nature of such clock, the exact temporal definition of a TTFL clock and their respective roles in early cell commitment are one of the hottest topics in the field.

THE TRUE COLORS OF CIRCADIAN PHOTOPIGMENTS

The nature of the photopigments and photoreceptors mediating mammalian SCN synchronization with the environment by means of light had been a puzzling issue until Provencio et al. (1998) discovered melanopsin in retinal tissues and hypotesized a role for it in circadian physiology. Then Hattar et al. (2002) and Provencio et al. (2002) went on to describe intrinsically photosensitive retinal ganglion cells (ipRGC), within the inner retina, containing melanopsin and sending monosynaptic projections to the SCN. For a long time the paradigm was that only these cells (about 1% of the all RGCs)–and not the classical photoreceptors rods and cones (contributing to perceptual vision and located in the outer portion of the retina)–contributed to photic entrainment of the master clock through their blue light-sensitive photopigment melanopsin. ipRGCs are less sensitive to light than rods and cones, they are depolarized rather than hyperpolarized by light, and more recently it has also been shown that, in addition to the SCN, they innervate several other areas of the brain, to regulate non-image forming responses to light (Fernandez et al., 2016). These include modulation of melatonin synthesis in the pineal gland, synaptic plasticity in the hippocampus (Fernandez et al., 2016) and functioning of the lateral habenula, which has been implicated in phenotypes such as sleep, mood and propensity to addiction (Baño-Otálora and Piggins, 2017).

More recent studies have pointed to an even more complex ipRGCs form of signaling to the brain, and to the SCN in particular, involving also rods and cones through largely unknown mechanisms but within a neural network which includes bipolar and amacrine cells (Ko, 2020). This model is supported by the observations that melanopsin null mutants mice can still be somehow synchronized by light (Panda et al., 2003) and exhibit phase shifting responses, albeit strongly attenuated (Ruby et al., 2002). Further, photic entrainment is abolished if ipRGCs are completely ablated (Chen et al., 2011). Thus it is ipRGCs and not melanopsin that are essential for photic entrainment, implying that they functionally interact with rods and cones. Therefore, alternative photopigments sensitive to other wavelengths could, through ipRGCs projections, modulate SCN photic entrainment and, most likely, also non-visual light responses depending on other areas of the brain. Finally, inner retina melanopsin, in spite of its low temporal resolution, has also been implicated in some features of form and spatial vision, raising fascinating questions on its role in visual perception (Allen et al., 2019). Thus, time seems ripe for un upgrade of the palette of light colors which modulate non visual photoreception. This will no doubt inform and possibly re-define some aspects of light hygiene over the 24 hours.

NON-CIRCADIAN RHYTHMICITY

Convincing evidence of the existence of *bona fide* endogenous clocks dictating time in temporal domains other than the circadian one, and information on their molecular and functional features are recent acquisitions. Evidence for circatidal, circalunar, circannual and seasonal biological rhythmicity is starting to acquire solid bases and some of the molecular components of these clocks have now been identified. Interestingly, there are indications that some canonical circadian clock genes also contribute to the generation of ultradian and infradian rhythmicity. Pioneering work in this respect has been performed in marine organisms such as *Euridice pulchra* (Zhang et al., 2013) and *Platynereis dumerilii* (Zantke et al., 2013) for which tidal and circalunar clocks have been shown to control tide-related migration and gonadal maturation, respectively. In the marine midge *Clunio marinus*, moon light seems to play an important role in circalunar clock synchronization (Kaiser et al., 2016). All these clocks also exhibit some degree

of independence, as they have been shown to function when the circadian clock is pharmacologically blocked (Zantke et al., 2013; Zhang et al., 2013). Further work is needed to define and functionally characterize the full set of components of such clocks, which represents one of the major current challenges in chronobiology.

In mammals, it has been observed that the phase differences between electrical and transcriptional/translational activity of neurons located in different SCN regions may reflect and thus code for the length of photoperiod (Inagaki et al., 2007; Yoshikawa et al., 2017; Honma, 2018). Such anatomical and functional organization may therefore provide organisms with relevant information to facilitate their adaptation to the environmental changes that characterize the course of seasons (circannual clock).

In humans, endogenous rhythmicity over any time scale is difficult to study, for reasons that are inherent to rhythms themselves and because of the masking/confounding effects of environmental cues to which we are sensitive, habits, social constraints etc. These can be removed only by complex and prolonged experiments [so-called constant routines (Duffy and Dijk, 2002)], which are generally performed in small numbers of young healthy individuals. Alternatively, rhythmicity can also be studied within the environment it is normally expressed in, in a sort of more ecological fashion, which yields somewhat less pure but still useful information. Further, while human circadian rhythmicity is fairly obvious, rhythms over different times scales are less apparent, and have not been the object of many studies. Of great interest, two sets of data have been recently published that provide examples of this non-circadian rhythmicity in humans. The first, which is the result of painstaking, patient and decades-long observations, has shown how women temporarily synchronize their menstrual cycles with the luminance and gravimetric cycles of the moon (Helfrich-Förster et al., 2021). The second one, which is the result of big data analysis, documents seasonality in human laboratory data collected for medical purposes, with a winter-spring peak in hormones related to reproduction, growth, metabolism, and stress adaptation (Tendler et al., 2021). It is not difficult to imagine how once the interest has focused on non-circadian human rhythmicity, both big data analyses of available datasets and the acquisition of new sets, for example by apps or other monitoring devices, will help producing information that is bound to be interesting and clinically relevant.

CHRONOBIOLOGY AND COVID-19

Infection from SARS-CoV-2 and the development of COVID-19 disease are very likely to affect circadian clock functioning. Moreover, rhythmicity over different time scales–most likely circadian and seasonal–may modulate the likelihood of acquisition and/or the course of infection and disease. Similarly, the intensive care arrhythmic environment may have unexpected effects on disease evolution (Haspel et al., 2021). The time of administration of approved treatments may impinge on the entity of their desired and side effects, in relation to both the nature of treatment itself, and to the patient's response to it (Haspel et al., 2021). These and other aspects of the complex and yet largely unexplored relationship between the pandemic and rhythmicity over different time scales have been considered by eminent colleagues in a collection of articles recently published in the Journal of Biological Rhythms (Sengupta et al., 2020, 2021; Borrmann et al., 2021; Cermakian and Harrington, 2021; Haspel et al., 2021; Kronfeld-Schor et al., 2021). As the pandemic continues to unfold, chronobiologists and scientists in related fields have become more sensitive to this relationship, and are examining available laboratory/clinical data retrospectively, and collecting them prospectively. Issues such as the appropriateness of time-stamping (clock time in addition to the full date) the acquisition of any human samples (may they be swabs, blood, urine etc.), the administration of treatment (Ruben et al., 2019) and the administration of vaccination for subsequent use in prognostic, large and long-term studies has once again come to the fore. Active, generous and powerful colleagues are lobbying on our behalf to this end.

Lockdowns put in place to different extents, in different countries and at different times of year have lead to some degree of stratification of society, with more fortunate groups enjoying some relief from social constraints and no major other changes to their lifestyle and productivity, and other groups being under considerable physical, emotional, organizational and economical stress, leading to a significant increase in mild and more severe psychiatric disorders (Holmes et al., 2020). There is an established relationship between psychiatric disease and rhythmicity, and the evidence for the benefits of chronotherapy (i.e., timed administration of light and/or melatonin) in this clinical field is considerably less anecdotal than generally perceived in other medical circles (Wirz-Justice and Benedetti, 2020).

At a time when all experience is needed to face the pandemic and its medium and long-term effects, translational chronobiology, chronopharmachology and chronotherapy, which also happens to be an inexpensive and substantially side-effect free form of treatment, may turn into powerful resources.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

PERSPECTIVE
published: 15 July 2020
doi: 10.3389/fphys.2020.00854

The Future of Physiology: Cardiac Electrophysiology

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Is cardiac electrophysiology complete? What are the challenges that are to be met in cardiac electrophysiology and how can we best engage these? These questions will be addressed in view of the progressing subspecialization of the field. A suggested answer lies in multidisciplinary and extradisciplinary approaches.

Keywords: cardiac electrophysiology, extradisciplinary research, multidisciplinary research, history, future

OPEN ACCESS

Edited by:
George E. Billman, The Ohio State University, United States

Reviewed by:
Crystal M. Ripplinger, University of California, Davis, United States
Vadim V. Fedorov, The Ohio State University, United States

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Specialty section:
This article was submitted to Cardiac Electrophysiology, a section of the journal Frontiers in Physiology

Received: *29 April 2020*
Accepted: *25 June 2020*
Published: *15 July 2020*

Citation:
Coronel R (2020) The Future of Physiology: Cardiac Electrophysiology. Front. Physiol. 11:854. doi: 10.3389/fphys.2020.00854

INTRODUCTION

Burdon Sanderson and Page wrote in 1879: "*. . .we owe most to the labours of Engelmann, whose researches . . . on the electromotive properties of the resting heart, and on the electrical changes which immediately follow excitation, leave little more to be done*" (Burdon-Sanderson and Page, 1879). They suggested that almost every question on cardiac electrophysiology had been answered and that the subject had been covered by their predecessor Engelmann (1877). Cardiac electrophysiology was complete. However, almost 120 years later the question whether cardiac electrophysiology was complete was addressed again by de Bakker in his inaugural lecture (de Bakker, 1999). de Bakker (1999) pointed to the many recent and new developments in the field and answered the question in the negative. There were still many challenges ahead in cardiac electrophysiology.

Indeed, after the publications of Burdon Sanderson and Page cardiac electrophysiology has flourished:

- Reentry was described as a potential mechanism underlying clinical arrhythmias (Mines, 1914).
- The Long QT syndromes were recognized as an entity (Jervell and Lange-Nielsen, 1957).
- The role of the autonomic nervous system on cardiac electrophysiology was studied (Schwartz et al., 1977; Shivkumar et al., 2016).
- The transmembrane current underlying pacemaking was discovered (DiFrancesco and Ohba, 1978).
- Ischemia-induced arrhythmias were mechanistically understood (Janse et al., 1980).
- Electrical cardioversion was successfully applied to stop lethal arrhythmias (Lown et al., 1986).
- The intricate relation between anatomy and function of the sinus and atrioventricular nodes was understood (Opthof, 1988; McGuire et al., 1996).
- The histology of myocardial infarction was linked to post-MI VTs (de Bakker et al., 1993).
- The coupling between cardiac mechanics and cardiac electrophysiology has been described (Franz, 1996; Kohl et al., 1998).
- The pulmonary vein myocardium was found as the origin of AF (Haissaguerre et al., 1998).
- The relation between embryological development and arrhythmias was established (Boukens and Christoffels, 2012).

Many more important and life-changing accomplishments of cardiac electrophysiology can be described here. As a spin-off of these insights, pharmacological and device therapies have been designed and new, -molecular biological- therapies are under development. The number of new inventions and breakthroughs have been almost endless and unanswered questions in cardiac electrophysiology remain. A few of these questions are enumerated in the table, some clinically relevant, others with a more basic science origin. The list is not intended to be complete or in order of relevance. Therefore, cardiac electrophysiology appears to have a future. However, is the subspecialization fit to address these questions?

THE DEVELOPMENT OF CARDIAC ELECTROPHYSIOLOGY AS A SUBSPECIALIZATION

Cardiac electrophysiology has a rich history (Lüderitz, 2002) and it is not easy to determine when cardiac electrophysiology constituted a distinct subspecialty of physiology and who was the first cardiac electrophysiologist. Burdon-Sanderson with his work on the cardiac currents resulting from local "injury" probably qualifies as the first cardiac electrophysiologist because he described what we now name a monophasic action potential or MAP (Burdon-Sanderson and Page, 1879; Franz, 1983). His pupil, Waller subsequently recorded the first electrocardiogram in man in 1887 (Waller, 1887). Thus, Burdon Sanderson and Waller shared and transferred their common interest in cardiac electrophysiology and thereby constituted the subspecialty. Nevertheless, the interest of these researchers was much wider than cardiac electrophysiology alone. They also studied the electrophysiology of nerves and plants (the fly trap), the effects of anesthesia and the influence of drugs on muscle contraction, to name but a few topics of their studies (Waller, 1887, 1910).

In the course of time, physiology have undergone further (sub)specialization. Physiology has developed into electrophysiology and cardiac electrophysiology (Weidmann, 1951). This course of (sub)specialization has followed the reductionist approach in science in general. In this approach the researcher uses progressively smaller and simpler models to exclude as many as possible confounding factors and to control all factors excepting the one that he/she is interested in. Indeed, at the same time cardiac electrophysiology evolved from the study of organisms (Waller, 1887) to that of organs (Mines, 1913) multicellular preparations (Kléber and Riegger, 1987) cells, expression systems, subcellular domains (Sanchez-Alonso et al., 2016; Rivaud et al., 2020), genes (Remme et al., 2006), or computer models (Shaw and Rudy, 1997; Hoogendijk et al., 2011). The reductionist development of scientific fields has also transpired in cardiac electrophysiology from which now a branch of cardiac *cellular* electrophysiology exists with dedicated textbooks and a European Working Group of Cardiac Cellular Electrophysiology (EWGCCE) that is part of the European Society of Cardiology. The identification of subcellular microdomains heralds the further subspecialization of the field in cardiac *sub*cellular electrophysiology.

The website of Frontiers in Physiology[1] is an example of the pedigree of physiology. It mentions 28 subspecialty sections amongst which are Autonomic Neuroscience, Biophysics, Cardiac Electrophysiology, Chronobiology, Clinical and Translational Physiology, Computational Physiology and Medicine, Embryonic and Developmental Physiology, Exercise Physiology, Integrative Physiology, Lipid and Fatty Acid Research, Medical Physics and Imaging, Membrane Physiology, and Membrane Biophysics. These subspecialties are not mutually exclusive. For example, studies on the Brugada Syndrome, a potentially lethal cardiac arrhythmia syndrome occurring at a relatively young age, are related to all of the above enumerated subspecialties of Frontiers in Physiology, but also to Frontiers in Cardiovascular Medicine (subspecialty Cardiac Rhythmology) (Antzelevitch et al., 2005; Hoogendijk et al., 2010; Zhang et al., 2015).

Although the reductionist approach is a powerful tool to identify mechanisms it has potential drawbacks. The results of reductionist studies cannot always be translated to more integrative levels of application. An example in cardiac electrophysiology is the use of the mouse as a model for human electrophysiology. Although the mouse is a good model for many mammalian characteristics including genetics, its ventricular action potential differs from that of man. Genetic studies on ventricular electrophysiology in mice therefore should be translated with caution to human electrophysiology (London, 2001). Another example is that of the use of a small portion of the ventricular wall as a model for the intact heart (Opthof et al., 2007).

The branching process (in progressively more subspecialities) facilitates but may also hinder engagement of the unmet challenges in cardiac electrophysiology. It is clear that within the domains of knowledge and techniques pertaining to cardiac electrophysiology we are more likely capable of finding an answer to the question "How does the Purkinje network relate to arrhythmias?" than to the question "How do we build a (human) heart?" (**Table 1**). Thus, it appears that physiology has branched into too many sub- and infra-specializations to allow a more generalist 'integrative' approach. How can we best address the unanswered questions?

INTEGRATIVE RESEARCH

In view of the immense growth of published knowledge in the course of the 20th century (Coronel, 2020) it would appear to be impossible to combine this knowledge in one person and to become a generalist again. Therefore, we need to resort to

[1]www.frontiersin.org

TABLE 1 | Some questions!

- How do we build a (human) heart?
- How can we prevent sudden death?
- How do we assess the risk of arrhythmias in patients?
- How do we diagnose and manage congenital arrhythmia syndromes?
- What is the relation between aging or prenatal development and arrhythmogenesis?
- Can we (non-invasively) map activation and repolarization of all myocardial components of the heart?
- What is the difference between the heart from a man and that of a woman?
- How can we best optimize the contraction sequence in diseased hearts?
- Do ion-transporting proteins have other (gene regulating, trafficking) functions?
- How does the Purkinje network relate to arrhythmias?
- What is the effect of obesity and other chronic diseases on arrhythmogenesis?
- What is the mechanism of J-wave syndromes?
- How do we prevent atrial fibrillation?
- Can we increase the total number of heart beats in a life?

collaboration with other scientists to complement the missing knowledge. Multidisciplinary research, the collaborative effort of scientists of various specializations and disciplines to solve a common problem, has been very successful.

Purkynje was the founder of the modern physiological laboratory and already was aware of the power of combining multiple disciplines (Travnickova, 1987). Multidisciplinarity was also applied by Dirk Durrer who combined the disciplines medicine, biology, biochemistry, electronics and physics (and a well-equipped workshop) for his research on cardiac electrophysiology and arrhythmias (Durrer et al., 1970). Many electrophysiological labs have followed this example and now also incorporate molecular biology, genetics, imaging, artificial intelligence, and developmental biology (stem cell biology) as disciplines to support electrophysiological research. The added value of the collaborations stems from the novel insights and techniques obtained in each discipline or from the discovery of new (genetic) syndromes (Early repolarization syndrome and Brugada syndrome). These insights/techniques encompass -amongst others- new model systems (for example, pluripotent stem cells), enlarged computing power, and new imaging modalities (scanning ion conductance microscopy, near infrared optical mapping in explanted human hearts) (Hansen et al., 2018). It remains a challenge to select the best possible fitting (animal) model to address a research also in more integrative modes of research. The best model for human (electrophysiology) remains the human, although this often is not ethically appropriate.

Multidisciplinary research potentially has an impact that reaches far outside the field of cardiac electrophysiology. In most examples of multidisciplinary research, the central research question or hypothesis is generated from a co-ordinating specialization to which the contributors from the other disciplines apply their knowledge. The question 'How do we diagnose and manage congenital arrhythmia syndromes?' can be solved by asking geneticists and molecular biologists to join a team of cardiologists.

Extradisciplinary research constitutes a particular mode of multidisciplinary research. This describes the scientific study of a research question or hypothesis generated in a different discipline (or subspecialty). Extradisciplinary research is a way to overcome the drawbacks of a too far progressed specialization and a way to a more integrative research approach of physiology. When cardiac electrophysiology was in its early days, the various disciplines were combined in a single person and extradisciplinary research was performed as a matter of course. A problem in plant physiology could thus be solved by applying knowledge from the mammalian heart and vice versa. Extradisciplinary input to cardiac electrophysiology can be anticipated from mathematics (inverse electrocardiography), developmental biology (congenital arrhythmia syndromes), and neurology (arrhythmia mechanisms). On the other hand, cardiac electrophysiology may impact on heart failure management (pacing), and cardiac repair by biological tissue constructs.

Cardiac arrhythmias are often associated with cardiac and extracardiac morbidities like obesity, hypertension, diabetes, atherosclerosis and neurological disorders. The exact contribution of these co-morbidities to cardiac arrhythmogenesis is often not known. A solution to these question lies in unraveling the interaction between extracardiac factors and cardiac electrophysiology by asking endocrinologists, immunologists and neurologists to address cardiac arrhythmogenesis and by stimulating cardiac electrophysiologists to perform extracardiac studies in extradisciplinary approaches.

Thus, if we wish to advance cardiac electrophysiology into the future, we need to allow a 'generalist' view on the subject by invoking other disciplines to operate on our turf. On the other hand, cardiac electrophysiology may well be fit to address questions on other fields of research.

CONCLUSION

Although cardiac electrophysiology has a rich history it is far from complete. There are still many questions that need to be resolved. However, cardiac electrophysiology has seen the emergence of subspecialties that may hinder a more generalist tackling of research. A plea is made for a multidisciplinary research and for extradisciplinary research in particular. As with all collaborative projects, the success of extradisciplinary research depends on mutual trust and clear communication between the scientists of the various disciplines.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

SPECIALTY GRAND CHALLENGE
published: 17 April 2020
doi: 10.3389/fphys.2020.00292

2020 Vision of Frontiers in Striated Muscle Physiology

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Keywords: striated muscle, future, skeletal, cardiac, physiology

Frontiers in Striated Muscle Physiology has published just over 400 articles, all online and freely accessible to everyone, since its inception in 2010. Of particular note is the fact that more than 75% of the published articles in this Journal have been submitted as contributions to Research Topics with clearly defined and unique themes. What is striking is that, with a primary focus on striated muscle physiology, a vast majority (two-thirds) of the 32 Research Topics to-date have included a focus, not necessarily singular, on skeletal or cardiac muscle disease, pathology or pathophysiology. Three other areas that have been well-represented by Research Topics are aging, muscle wasting and fatigue/weakness. The remaining 25% of Research Topics had a primary focus on fundamental properties of striated muscle. Therefore, this Journal has been, and likely will remain, an important venue for the dissemination of a substantial amount of cutting-edge knowledge to better understand not only fundamental striated muscle physiology but also conditions that lead to significant decrements in muscle function. All of this success stems from the hard work of the authors and the associate editors, guest editors, and review editors who collectively represent a broad community of international scientists with an interest in open-access publishing.

Looking ahead for what are likely trends in the field of striated muscle physiology, several areas standout based upon what has evolved over the past decade. There has been a clear surge of studies that were focused on sex differences in virtually all physiology systems, including skeletal muscle and the heart, over the past 10 years and many published reports have revealed sex-related differences in the human population, as well as in model organisms. The results of a PubMed search with "muscle sex difference" as a search term, indicates a peak in the number of results in 2009, followed by a 3-year period with a small decrease in the number of annual publications. Beginning in 2013 there was a clear jump in the number of results, with a fairly steady increase through 2018 (the indicated number was lower for 2019 when the search was conducted and that might have been due to an incomplete compilation of results at that time). The increase in the number of published articles on sex-related differences coincides with an awareness of the importance of the inclusion of both sexes in the design and execution of studies of all physiological systems.

A more complete understanding of mechanisms that contribute to the maintenance of, or decrements in, muscle mass in association with aging, myopathies, and other diseases, such as cancer and AIDS, has been a goal for several decades and it is imperative that this remain a major focus well into the future. Not only is a loss of muscle mass and strength a major concern for many individuals, the physiological changes in muscle with reduced mass and the impact on recovery from muscle injury are not well-understood. A major goal should be understanding how muscle mass can be maintained during normal aging and in the context of specific myopathies and, more generally, in association with a broad spectrum of diseases.

It is well-recognized that skeletal muscle and cardiac muscle have the potential to express a myriad of sarcomeric protein isoforms that change during the course of normal development, in association with disease and following skeletal muscle injury and myocardial infarction. Given the large number of genes that code sarcomeric proteins that are expressed in striated muscle and the extensive alternative splicing that is known to occur in striated muscle, the number of possible

OPEN ACCESS

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Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: *09 March 2020*
Accepted: *16 March 2020*
Published: *17 April 2020*

Citation:
Reiser PJ and Janssen PML (2020) 2020 Vision of Frontiers in Striated Muscle Physiology. Front. Physiol. 11:292. doi: 10.3389/fphys.2020.00292

combinations of expressed isoforms and, therefore, of the potential number of fiber types, is extraordinary. Yet it is also well-recognized that there is a limited number of fiber types that actually exist in striated muscles. How coordination of gene expression is executed to yield a very complex but finite number of combinations of sarcomeric protein isoforms, that directly regulate contractile properties of skeletal and cardiac muscle, is not well-understood. Elucidating the mechanisms that coordinate sarcomeric protein expression, given the impact on striated muscle function, should remain a major focus of investigation.

The study of the impacts of environmental changes, such as global warming and a greater prevalence of air pollution in cities, on many physiological systems, especially cardiovascular, and respiratory, has been intensely studied. However, with the likely persistence and worsening of these changes, rigorous investigations of the impact on not only the human population, but on all organisms, must remain as an elevated priority.

One area of investigation that is clearly on the rise is skeletal muscle exosomes—released extracellular vesicles. A PubMed search for "muscle exosomes" generated fewer than 10 hits annually, prior to 2013. Since that year, the annual number has grown steadily, reaching 127 in 2018. This is an area that is expected to grow substantially as more is learned about the contents of muscle exosomes, their targets and roles in pathophysiology. A review of skeletal muscle exosomes, with a focus on their involvement in metabolic diseases, was recently published in this Journal by Rome et al. (2019). The authors identified several areas in which more studies are needed to better understand the role of muscle exosomes in health ad disease. A very similar surge is found in a PubMed search for "cardiac exosomes." More than half of all the hits in PubMed have been published in just the last 2 years. Combined, striated muscle exosomes point to a rapidly developing field that is indicating a role that striated muscle plays in the possible regulation and tuning of other systems in the body.

An additional area in which substantial advancement is anticipated in the near future is the utilization of high resolution cryo-electron microscopy. Given the inherent highly organized intracellular structure of striated muscle, the three-dimensional relationships of organelles and proteins to each other is critically important for proper function. Cryo-EM has the potential to yield an understanding of the precise structural relationships among intracellular components with near atomic resolution. There is a sense that exciting discoveries are inevitable with this imaging modality.

Molecular dynamics is also an emerging tool that is seeing a rapid increase of use in the striated muscle field. The computational analysis of each atom within a protein over time allows the study of protein interactions at the very single molecule level. Although currently limited to small time domains, and to the interaction of only a few proteins, with increased computational speed, eventually more complex systems, such as the sarcomere, can be modeled. With further enhancements in computational power, we should eventually be able to go through a contraction cycle, allowing us to learn the molecular nuances that make this intriguing sarcomere structure function. In addition, this will allow us, likely in combination with cryo-EM, to discern the impact of single amino acid mutations.

Rapid growth is also seen in the study of post-translational modifications (PTMs). This is of particular interest for proteins involved in the contractile aspects of striated muscle, as the vast majority of muscles consists of sarcomeric proteins that are stoichiometrically expressed. Thus, regulation of function does not necessarily take place via the amount of proteins expressed, unlike processes such as ion channels, and cell signaling that are predominantly regulated by the amount of protein. Since the contractile proteins are expressed at a fixed ratio, the majority, if not all, of the regulation of the function of these proteins relies on post-translational modifications. A challenge is to capture these modifications in their dynamic state, as several of these PTMs are labile, and can rapidly occur or dissipate. For instance, beta-adrenergic stimulation results in key phosphorylations in several proteins involved in contractile regulation, including phospholamban and troponin-I. This phosphorylation process occurs in mere seconds, or even faster, and constitutively active phosphatases can de-phosphorylate also in a very short time frame. Reliably capturing and analyzing the levels of PTMs will therefore be a challenge, but when achieved likely will yield a high pay-off in providing critical data on regulation of striated muscle function.

The past 10 years has been a period of great success for this Journal, in terms of the quality and scope of articles that have been published. Since 2011, the Journal has been growing on average 37% per year, with nearly 550 submissions to-date, and a 60% growth in manuscript submissions in the last 6 months, compared to the preceding 6-month period. Much of this success stems from the engagement of authors in response to *Research Topics*. Suggestions for future topics are welcome from all readers and may be submitted to either of the authors of this Perspective for initial consideration and potential development.

AUTHOR CONTRIBUTIONS

PR and PJ wrote the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

SPECIALTY GRAND CHALLENGE
published: 07 August 2020
doi: 10.3389/fphys.2020.00852

Frontiers in Vascular Physiology Grand Challenges in Vascular Physiology

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Keywords: vascular, cardiovascular, medical, vascular physiology, circulation

We have reached a new decade and have witnessed a plethora of remarkable advances in biology and medicine over the last decade. Importantly, the 2020's certainly promise to deliver even more exciting innovations than the last decade as we continue to integrate molecular and cellular data into a more comprehensive understanding at the tissue, organ, system, and organismal levels. For vascular physiology and medicine these are especially exciting times. We have witnessed rapid advances in vascular biology-related genetic biology, technological advancements in the bioengineering of organs and tissue, improvements in instrumentation and technology, progress in robotics, and artificial intelligence to name but a few. The principal objective of this paper is to highlight key recent achievements, future possibilities and the need for continued advances in the field of vascular physiology.

We have a continuing need for investment of resources in research directed at cardiovascular disease (CVD). As a non-communicable disease, morbidity and mortality from cardiovascular pathology is one of the major health issues that impacts the quality of life and contributes to premature death. Despite a reduction in CVD related mortality as a consequence of developments in both medical and surgical interventions (Mensah et al., 2017), this remains a worldwide health problem with immense economic impact. Using data provided by the American Heart Association for the United States as an example, the cost of CVD and stroke was estimated at $555 billion for 2016 while being projected to rise to over $1 trillion by 2035 (American Heart Association, 2017; Benjamin et al., 2019). These figures rise considerably if costs related to lost productivity and nursing homes are estimated. Impact fact statements for the UK (UK Fact Sheet, 2019) estimating yearly costs of CVD at 19 billion GBP and multiple billions of Euros across Europe (Timmis et al., 2020) are a further testimony to the negative effects of CVD on our society. Almost certainly similar data exists globally, particularly with the "westernization" of developing countries. These are staggering and sobering figures that underscore the need for continued research into the risk factors that predispose toward developing CVD and into the mechanistic causes and therapeutic approaches to treatment and management. Based on this economic and health related data it is understandable why there has been a shift toward translational/clinical relevance however, this must not exclude fundamental research in cardiovascular physiology which remains an essential approach to understand important disease processes.

In the coming decade will see increasing challenges in the cardiovascular health field, particularly as rates of insulin resistance, obesity and overt type II diabetes continue to rise (Ward et al., 2019). In this regard, in the United States, obesity (BMI $>$ 30) and severe obesity (BMI $>$ 35) are predicted to reach $\sim$50 and 25% of the population, respectively, by 2030 (Ward et al., 2019). Similarly, the cardiovascular impact of exposure to particulate matter is being more and more appreciated. Increasing evidence is accumulating that exposure to airborne inhalable (PM_{10}) and fine ($PM_{2.5}$) particles predispose to cardiovascular disorders including those that are independent of pulmonary effects (Hamanaka and Mutlu, 2018; Zhang et al., 2018; Combes and Franchineau, 2019). Thus, exposure to particulate matter has been linked to increased prevalence of metabolic disorders and even to predisposing the developing fetus to vascular disease in later life. Recently, the cardiovascular impacts of electronic cigarettes, or "vaping" is also

OPEN ACCESS

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Specialty section:
This article was submitted to Vascular Physiology, a section of the journal Frontiers in Physiology

Received: *09 March 2020*
Accepted: *24 June 2020*
Published: *07 August 2020*

Citation:
Meininger GA and Hill MA (2020) Frontiers in Vascular Physiology Grand Challenges in Vascular Physiology. Front. Physiol. 11:852. doi: 10.3389/fphys.2020.00852

now on the horizon. Further, cardiovascular disorders have emerged as a result of long-term medical treatments (e.g., cardiotoxicity of anti-cancer agents) and increased longevity in patients with chronic conditions (e.g., Duchenne's Muscular Dystrophy and cardiomyopathy). As we have realized over the last decade, there is an increasing rate of CVD driven by the Westernization of developing countries. As mentioned above, this trend continues. Finally, the current SARS-CoV2 (Covid-19) pandemic highlights the multiple interactions that can occur between an infectious viral disease and the cardiovascular system. Although early in our full understanding of the pathogenesis of Covid-19 infection, the cardiovascular system contributes to cellular viral entry via the angiotensin converting enzyme 2 (ACE2) (Wan et al., 2020) while also being directly impacted by the virus (for example as reflected in myocarditis and clotting abnormalities) (Libby, 2020; Spiezia et al., 2020). Further, severity of Covid-19 infection, in terms of negative outcomes, is negatively affected by multiple factors impacting the cardiovascular system including co-existing obesity, cardiometabolic disease, diabetes and health disparities (Belanger et al., 2020; Hill et al., 2020; Muniyappa and Gubbi, 2020; Wu and McGoogan, 2020).

These CVD statistics are certainly dramatic but looking forward there are many ongoing developments in the field that will have significant impacts on cardiovascular research and health over the next decade. Imaging continues to be a technology-based shining star. Consistent with the adage, "if you can see it you can study it," new developments in imaging are continuing to emerge. In the world of optical microscopy we are seeing improvements in resolution that are allowing visualization of molecular events, new fluorescent agents are improving selective targeting and microscope automation is driving large increases in throughput. Such improved modes of imaging include super resolution, light sheet microscopy with 4D capability as well as increased application of optical techniques such as cryo-electron microscopy. The increased availability of these approaches will benefit the cardiovascular field. In the last decade we have also witnessed a rapid growth in the use and application development of techniques involving scanning probe technology, for example, atomic force microscopy. These techniques have proven their usefulness in allowing single molecular events to be studied and to biomechanically probe cells and molecules. Rapid advances in high speed atomic force microscopy have advanced this technology even further into the realm of observing of molecular events and it will be exciting to see further developments allowing reliable live cell applications to become a reality. In cardiovascular research we need further development of applications to permit visualization of the vasculature at all scales of organization from large conduit vessels to the smallest vessels of the microcirculation. Techniques for measurements of vascular dimensions, perfusion, circulating cell distribution, and vessel wall biomechanics to name a few are all critical parameters for the study of organ function in health and disease. As the endeavors of developmental biology, regenerative medicine, organ transplantation, and artificial organs advance the ability to monitor the functioning vasculature is key for the success of these endeavors.

Over the coming decade there will be an increased availability of human data through electronic medical records and commercial sources. Tissue obtainable through biorepositories, increased access to patient databases and genetic screening, non-invasive ambulatory technologies that monitor health, drug delivery and communicate with doctors are all examples of new emerging sources of material for research. With all of these technologies and the automation in computer driven data collection there has been a tremendous increase in the size of data sets. Multi megabyte and even terabyte data sets are becoming more common, the magnitude of which is beyond human capacity to critically evaluate such results. Thus, there is a critical demand for improvements in bioinformatics and data processing that will ease user interaction with these large data sets and permit the objectivity needed to extract essential features for accurate hypothesis testing.

Over the last decade there has been a continued emphasis on reductionism as we strive to understand biological function even more deeply. As we look forward at ways to interpret the relevance of this information there is a necessity for continued development of non-invasive and minimally invasive forms of imaging and biosensing that will allow study of physiological parameters and biochemical events in intact animals and subjects. In this regard, a noticeable shift in research strategy during the last decade has been to complement cellular and molecular approaches with sophisticated *in vivo* techniques. Of particular significance are approaches allowing temporal analysis of disease models as provided by imaging techniques (MRI, SPECT, high frequency ultrasound and photoacoustics, telemetry), metabolic phenotyping, and behavioral analysis. These are very encouraging developments that will help to promote a more integrative view and improved accuracy of interpretation culminating in a more integrative approach to understanding cell molecular data. Physiology is, after all, a field that strives to understand the intact functioning system at the organ and organismal level. It equally important we see these trends extended to an educational level where a resurgence of some classical discipline-based training is important and should be given increased priority. The growing need for research using whole animal and organ level experimental research design requires an increasing not shrinking population of physiologists with the skills to perform these types of experiments.

Another area that should not be overlooked in the coming decade is continuing to build our skills in modeling biological systems. The ability to produce accurate mathematical models of complex data and complex system behavior is proving an important partner in hypothesis generation and testing and data interpretation. Models provide the ability to produce physical representations underlying experimental results. This allows novel insight into data and provides a means for generating testable new hypotheses. Some examples include, understanding perfusion patterns, nutrient distribution, distribution of mechanical forces within the vascular system, vascular remodeling and development, drug delivery and targeting, and molecular behavior underlying cell and vessel scale behaviors. Thus, modeling contributes greatly to our understanding of cardiac and vascular function by significantly

TABLE 1 | Top ten articles downloads (upper table) and citations (lower table) from Frontiers in Vascular Physiology in the last decade.

Title	DOI	Views	PDF Downloads
MicroRNA regulation of SIRT1	10.3389/fphys.2012.00068	11,878	5,207
Role of tumor associated macrophages in tumor angiogenesis and lymphangiogenesis	10.3389/fphys.2014.00075	16,268	5,207
The CXCL12/CXCR4 chemokine ligand/receptor axis in cardiovascular disease	10.3389/fphys.2014.00212	20,178	4,967
Transforming growth factor-β and endoglin signaling orchestrate wound healing	10.3389/fphys.2011.00089	15,844	4,860
Caveolae, caveolins, cavins, and endothelial cell function: new insights	10.3389/fphys.2011.00120	11,259	4,547
Effects of exercise on cardiovascular performance in the elderly	10.3389/fphys.2014.00051	24,669	4,114
Macrophage phenotype modulation by CXCL4 in atherosclerosis	10.3389/fphys.2012.00001	12,129	3,897
Vascular inflammatory cells in hypertension	10.3389/fphys.2012.00128	11,986	3,871
Mesenchymal stem cell-derived extracellular vesicles promote angiogenesis: potential clinical application	10.3389/fphys.2016.00024	12,319	3,776
Molecular pathways of notch signaling in vascular smooth muscle cells	10.3389/fphys.2012.00081	10,721	3,578

Title	DOI	Views
Role of tumor associated macrophages in tumorangiogenesis and lymphangiogenesis	10.3389/fphys.2014.00075	16,268
Caveolae, caveolins, cavins, and endothelial cell function: new insights	10.3389/fphys.2011.00120	11,259
Mechanisms involved in the aging-induced vascular dysfunction	10.3389/fphys.2012.00132	8,830
The CXCL12/CXCR4 chemokine ligand/receptor axis in cardiovascular disease	10.3389/fphys.2014.00212	2,017
Vascular inflammatory cells in hypertension	10.3389/fphys.2012.00128	11,986
The role IL-1 in tumor-mediated angiogenesis	10.3389/fphys.2014.00114	9,407
MicroRNA regulation of SIRT	110.3389/fphys.2012.00068	11,878
Vulnerability of the developing brain to hypoxic-ischemic damage: contribution of the cerebral vasculature to injury and repair?	10.3389/fphys.2012.00424	13,838
Mesenchymal stem cell-derived extracellular vesicles promote angiogenesis: potential clinical application	10.3389/fphys.2016.00024	12,319
Macrophages and chemokines as mediators of angiogenesis	10.3389/fphys.2013.00159	10,289

expanding the number of potential interacting variables that can be investigated and dynamically tracked. Searching for single variable answers in complex and dynamic systems is often a flawed approach to the complex hypotheses we are attempting to test. Further, the availability of approaches for handling large data sets will be critical for the development and implementation of powerful machine learning approaches for the study of complex cellular signaling networks relevant to cardiovascular tissue and disease processes (Ping et al., 2018).

Although the next decade looks extremely promising we have unfortunately seen the last decade present some real threats to the quality of cardiovascular research and biomedical research, in general. Specifically, it is worth mentioning that as scientists we are facing a time when we have to exercise increased ethical vigilance when we select journals to publish in and scientific meetings to attend. The integrity of the field is now impacted by the proliferation of journals that seem to be driven by profit rather than the science. Many of these have been referred to as "predatory journals" as typified by Beal's List and discussed in a number of editorials/commentaries (Strielkowski, 2018; Grudniewicz et al., 2019). Journals that do not adhere to the reputable peer review codes and that have no societal or academic affiliations. Whether the reason for this trend is fueled solely by profit or also by pressure to publish for promotion and advancement is unimportant when you consider the increases in the likelihood that unreliable and non-reproduceable data are entering the scientific and public domain. Paralleling the proliferation of journals is the surge in invitations we receive to

serve on editorial boards and be associate editors for journals beyond our qualifications and to attend scientific meetings without merit or the backing of any legitimate scientific society or academic organization. These trends not only threaten the integrity of our field but they are dangerous when one considers that this can unduly influence the lay public and government. Collectively, in our opinion these trends do not facilitate the advancement of science.

Also worthy of further comment is that over the last decade the biomedical research community has increasingly questioned why published studies are often difficult to replicate. Sometimes even within the same laboratory. Importantly this has prompted increased attention being given to the fidelity and source of reagents (e.g., specificity of antibodies, genetic constructs) and experimental models (genetically engineered animals and cells for tissue culture). While this topic has now been the subject of many editorials and this issue is being embraced by funding agencies (for example, see NIH guidelines for rigor and reproducibility www.nih.gov/research-training/rigor-reproducibility), reputable journals, discussion boards and individual scientists it is an issue that will require continued consideration by the community and by the individual scientist.

Reviewing the last 10 years of Frontiers in Vascular Physiology it has been interesting to examine/study the trends in the field. Some insights can be seen by looking at published topics that have received the most attention in terms of downloads and citations. These are summarized in the following tables (**Table 1**). Importantly, the Frontiers in Vascular Physiology is publishing studies that are contributing to our knowledge of vascular biology spanning basic, translational and therapeutic topics. Further, our publications reflect the global community of vascular physiologists. With the advances in vascular physiology anticipated for the next decade the future of the Journal seems equally promising and will continue to be a forum for us all to witness the continued progress of the field.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

SPECIALTY GRAND CHALLENGE
published: 08 March 2021
doi: 10.3389/fphys.2021.650503

Diabetic Kidney Disease Represents a Locus of Opportunity

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Keywords: diabetes, kidney, diabetic nephropathy, hypertension, renal physiology and pathophysiology

According to the World Health Organization, the global presence of type 2 diabetes (T2D) rose sharply from 4.7 to 8.5% of the population between the years 1980 and 2014 (Emerging Risk Factors Collaboration et al., 2010). Moreover, T2D is associated with a 30–50% risk of diabetic nephropathy (DN) (Gheith et al., 2016). Given a world population of ∼7.8 billion (December 2020) (Worldometer, 2020), we may expect as many as 300–400 million affected persons. DN is the leading cause of end-stage renal disease (ESRD) (Toth-Manikowski and Atta, 2015). Currently renal transplantation is the treatment of choice for patients with ESRD due to improvements in graft survival; however, the wait for an available organ may extend to 3–5 years (Allen et al., 2018; Clayton et al., 2018). Dialysis is clearly not the answer, at least as it is currently employed, as the 5-year survival rate for patients receiving some form of hemodialysis hovers only at around 20–40% (Huff, 2020). Moreover, the cost of treating these subjects is phenomenal, and many cannot gain access to or afford any type of treatment. Only a handful of new innovative and efficacious strategies to combat DN has made it to the clinic in the last 50 years. Why is this the case?

It is partly because we do not fully understand the problem. The kidney is clearly a complicated organ derived from three overlapping sequential systems—the pronephros, the mesonephros, and the metanephros, which are all derived from the urogenital ridge (Qiaggin and Kreidberg, 2006). The kidney conducts a variety of seemly unrelated tasks utilizing a variety of specialized cell types extending along the renal tubule. The 3-D architecture of the kidney is essential in its role in regulating whole-body fluid balance, acid-base homeostasis, blood pressure control, excretion of toxic substances, and reabsorption of vital filtered substances. In the process of cleaning the blood, the proximal tubule (PT) is tasked with reabsorbing and recycling a number of substances, e.g., glucose, amino acids, electrolytes, and water, which would otherwise be lost. Urine is concentrated by the use of energy to generate a cortico-medullary sodium and urea gradient in the interstitium, allowing for passive reabsorption of water, regulated tightly by vasopressin. All of these aspects of normal kidney function can be compromised by DN.

DN may be described as a "perfect storm" involving inflammation, fibrosis, and oxidative stress. Histologically, it has a number of features that distinguish it from other forms of renal disease. For example, Kimmelstein-Wilson lesions, composed of nodular, circular, scar tissue, will form in the glomerulus. Other features include an expanded mesangium and increased mesangial cell number in the glomerulus. The basement membranes of the glomerulus and tubules become thicker (up to 3X) with deposition of collagen, albumin, and IgG along their borders. There is an accumulation of the advanced glycation end product (AGES) due to the partially reduced sugar moieties. AGES increases protein cross-linking, inflammation, and oxidative stress. Foot processes in the glomerulus merge. A common theme underlying the pathology of DN can be broadly thought of as dysregulated utilization of energy.

We can treat DN both by treating T2D itself, i.e., tightly controlling blood glucose levels, as well as, via strategies that short-circuit the effects of T2D on the kidney. For purposes of this article, I will focus primarily on the latter. First, I will discuss the specific challenges of DN in regard to mechanisms of the disease, and standards of care. Second, I will discuss some newer medication strategies that are promising. Finally, I will turn to developing approaches to treat the patient when

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Renal and Epithelial Physiology, a section of the journal Frontiers in Physiology

Received: *07 January 2021*
Accepted: *01 February 2021*
Published: *08 March 2021*

Citation:
Ecelbarger CM (2021) Diabetic Kidney Disease Represents a Locus of Opportunity. Front. Physiol. 12:650503. doi: 10.3389/fphys.2021.650503

the kidneys have failed, e.g., xenotransplantation, organ regeneration, and portable artificial kidneys.

T2D is associated with a number of physiological changes, the most obvious being hyperglycemia. One of the main targets of hyperglycemia in the kidney, as well as other organs, is the vasculature (Magee et al., 2017). Endothelial dysfunction, impaired nitric oxide generating capacity, as well as, atherosclerotic plaque formation contribute to impaired blood flow and altered renal hemodynamics. Hyperglycemia is also associated with hyperinsulinemia early in the course of T2D (Tiwari et al., 2007b). We have shown insulin receptor expression is reduced in the cortex and medulla of the kidney in hyperinsulinemic, obese, and T2D rats (Tiwari et al., 2007a). Nonetheless, some aspects of insulin signaling may remain intact in the kidney, in particular those acting through the insulin receptor substrate type 2 (IRS-2) (Ecelbarger, 2020). This can lead to inappropriate upregulation of sodium reabsorption and gluconeogenesis in the PT (Nakamura et al., 2015, 2019).

In the kidney *per se*, the metabolism of epithelial and endothelial cells is altered in an environment in which glucose levels are higher than the norm, i.e., 5.5 mM. Elevated cellular glucose levels lead to a rise in oxidative phosphorylation by the mitochondria. This liberates oxidative radicals including superoxide, which may overwhelm the normal anti-oxidative complement of the mitochondria, e.g., manganese superoxide dismutase (Burgos-Moron et al., 2019). Reactive oxygen species (ROS) can damage cellular DNA, lipids, and proteins, as well as other organelles, such as the endoplasmic reticulum (ER). It appears that mitochondrial DNA, which codes for many of the components of the electron transport chain, is particularly susceptible to ROS damage (Burgos-Moron et al., 2019). Damage to the ER can lead to misfolded proteins, a critical step in their biogenesis (Zeeshan et al., 2016).

Inflammation in DN is initiated as a protective response to early tissue injury or cell death (Perez-Morales et al., 2019). Major players in the inflammation associated with DN (which can include both systemic and localized aspects) include activation of macrophages, the nuclear transcription factor (NFκb), the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, and inflammatory cytokines. Inflammatory cytokines include interleukins-1, -6, 18, and tumor necrosis factor, alpha (TNF-α). Chemokines, which attract other inflammatory molecules as part of their actions, include monocyte chemotactic protein-1 (MCP-1) and a number of chemokine C-C motif ligands, e.g., CCL2, CCL5, and CX3CL1. There are currently a number of anti-inflammatory approaches to treat DN in various stages of clinical trials (Perez-Morales et al., 2019). For example, pentoxifylline (PFA) targeting cytokines (Navarro-Gonzalez et al., 2018), baricitinib, a JAK 1/2 inhibitor (Tuttle et al., 2018), CCX140-B, a CCR2 inhibitor (de Zeeuw et al., 2015), and emapticap pegol, an MCP-1/CC2 antagonist, have all been shown to reduce albuminuria in separate clinical studies (Menne et al., 2017).

Hypertension is a major risk factor for the development of DN (Lovshin and Cherney, 2014). Hyperglycemia likely affects blood pressure through a number of indirect means. Increased pressure at the level of the glomerulus causes podocyte effacement and, eventually, nephron dropout, which consequently increases the load on remaining nephrons. In line with this, inhibitors of the renin-angiotensin-system (RAS) are the current medical "therapy of choice" for the treatment of DN. Several clinical trials have demonstrated a protective effect to reduce albuminuria in hypertensive/proteinuric patients as the result of treatment with angiotensin-converting-enzyme inhibitors, e.g., captopril or angiotensin receptor blockers (ARBs), e.g., losartan (Brenner et al., 2001; Amann et al., 2003). Dual treatment with both classes of drugs did not seem to provide any additive effect (Imai et al., 2013). Other more recent drug combinations, such as aliskiren (renin blocker) with ACEs or ARBs, did not show added benefit and may even have been harmful (Cully, 2013).

Hyperfiltration, supraphysiologically elevated glomerular filtration rate, i.e., GFR >135 ml/min/1.73m^3, is found to occur early in the course of T2D (Tonneijck et al., 2017), and advances DN. Hyperfiltration may be due to an influx of amino acids in the renal circulation (especially those that promote gluconeogenesis) (Tuttle and Bruton, 1992) or the result of altered tubuloglomerular feedback (TGF), i.e., increased glucose reabsorption in the PT leading to increased sodium reabsorption at this site (Vallon and Komers, 2011). The net result is less sodium at the macula densa, which releases neurohumoral factors via the juxtaglomerular apparatus (JGA), resulting in changes in the pre- or post-glomerular arteriolar tone (Tonneijck et al., 2017). This increases GFR and allows for some sodium to travel to the distal tubule. Other factors present in T2D that may alter the glomerular arteriolar tone include insulin, cyclooxygenase 2 (COX2), angiotensin II, and atrial natriuretic peptide. Despite an abundance of evidence that hyperglycemia is upstream of a plethora of pathways that have been shown to be damaging to the kidney, there is little evidence to show that strict glucose control alone, in the clinical setting, can reverse pre-existing DN (Wong et al., 2016).

SGLT2 (sodium glucose transporter, type 2) inhibitors represent a surprising and major breakthrough in the treatment of DN (DeFronzo et al., 2017). The SGLT2 protein is expressed in the brush border of the S1–S2 portions of the proximal tubule, and considered to be a high-capacity, low-affinity transporter (Poulsen et al., 2015). It appears, somewhat in series, with the SGLT1 isoform, in the S2–S3 segments, and not a target for the inhibitors. The original aim of this drug class, as developed, was to design a safer drug with similar properties to Phlorizin (blocked both isoforms, but which had intestinal side effects) (Rieg and Vallon, 2018). The goal was to deplete circulating glucose levels in the blood by blocking its reabsorption in the kidney; however, the drugs have subsequently been demonstrated to have protective actions independent of their ability to reduce blood glucose, effects that are not clearly understood. A recent meta-analysis to assess cardiovascular and kidney outcome of all 4 available SGLT2 inhibitions, i.e., canagliflozin, empagliflozin, dapagliflozin, and ertuglifozin, in T2D patients demonstrated a consistent improvement in kidney composite outcomes (Hazard ratio = 0.62) across the class (McGuire et al., 2021).

One challenge to overcome is that it is difficult to diagnose DN in its most nascent stages. The presence of microalbuminuria (30–300 mg albumin excreted/day) is an early indicator not only of the presence of renal compromise but also correlates to increased cardiovascular risk (Xia et al., 2015; Al-Rubeaan et al.,

2017). Unfortunately, the presence of microalbuminuria is less predictive of the progression of DN in T2D, as compared to T1D (Molitch et al., 2004). There is a need to develop and validate more sensitive biomarkers. The presence of certain microRNAs in urine exosomes may be promising to detect early cellular stress and alterations in fibrotic and autophagic pathways (Ma et al., 2019). Other promising protein-based biomarkers include serum cystatin C (to estimate GFR), urinary angiotensinogen and ACE2, plasma copeptin, serum amyloid A, and serum and urinary zinc α2 glycoprotein (ZAG) (Colhoun and Marcovecchio, 2018).

Other new areas of research include finding and testing direct targets of hyperfiltration, inflammation, and fibrosis (Alicic et al., 2017). A protein kinase C (PKC) inhibitor, ruboxistaurin, was found to reduce albuminuria in T2D (Tuttle et al., 2005). Niclosamine, developed originally as an antihelmintic (tapeworm medicine), is currently in the recruiting stage for a clinical trial for DN (Chen et al., 2018; Mook et al., 2019). Underlying its mechanism of action includes uncoupling of oxidative phosphorylation, and modulation of the Wnt/β-catenin, mTORC1, STAT3, NF-κB, and Notch signaling pathways (Chen et al., 2018). Other new drugs being tested in clinical trials include magnesium supplementation and LMB763, a farnesoid X receptor agonist (U.S. National Library of Medicine, 2020).

There are also technological advances on the horizon for those needing renal replacement therapy (RRT) or those with ESRD (Dang et al., 2020). One area of research is in non-cell-based kidney replacement primarily in the form of wearable artificial kidneys (WAK) and implantable artificial kidneys (IAK) (Salani et al., 2018). Technologically, there were a number of barriers to overcome in making these devices preferable to conventional dialysis at a center, e.g., the volume of fluid needed, power requirements, and weight. Wearable devices in clinical trials currently are $<$5 kg, and this is accomplished by recycling the fluid using a column that contains urease to hydrolyze urea into ammonia and carbon dioxide (Salani et al., 2018). IAK have not yet advanced beyond animal model systems. The current system in development uses cardiovascular pressure and chemical energy of cellular metabolism to derive power. Also, no dialysate is required as patients consume an electrolyte-rich fluid. Limitations include the invasive procedure to implant the device, and the lifespan of each device is uncertain.

Another possibility gaining traction is to develop a biological kidney prototype, or a tissue-regenerated kidney (Dang et al., 2020). Bioengineered kidneys are generally designed by applying cells to a biologic or partially synthetic scaffold so that the cells, hopefully, given the right spatial cues, reseed and grow a filtering, homeostatic, device similar to the native kidney. Success at this level would be wonderful and assuage the global demand for RRT; however, this may be a ways off, as the kidney is a highly complex organ with more than 26 specialized cell types (Nishinakamura, 2008). A perhaps more realistic short-term approach involves xenotransplantation of "humanized" organs from transgenic animals (e.g., pigs; Peired et al., 2020); however, ethical issues surround this approach.

In conclusion, DN represents a complex disease. It is not only difficult to detect in the early stages, but it is difficult to slow once it is initiated. While dietary management (low Na+, K+ diets) and ACEs and ARBs have been demonstrated to slow the progression, the staggering numbers of currently affected individuals worldwide, as well as those not able to access even these basic therapies, indeed make it a Grand Challenge in the kidney physiology field. We need to dream big and challenge ourselves to find novel approaches to address this often silent killer.

AUTHOR'S NOTE

Grand Challenge, authored by the Specialty Chief Editor of the Renal and Epithelial Physiology section.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

FUNDING

CE is supported by Georgetown University Department of Medicine and NIDDK (AWD-7774031, Chronic Kidney Disease (CKD) Biomarkers Consortium, GR411670 sub-contract to UPENN).

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

SPECIALTY GRAND CHALLENGE
published: 19 May 2021
doi: 10.3389/fphys.2021.689429

Interleukin-6 Trans-signaling: A Pathway With Therapeutic Potential for Diabetic Retinopathy

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Keywords: interleukin-6, IL-6 trans-signaling, sgp130Fc, inflammation, diabetic retinopathy

NEW THERAPIES FOR DIABETIC RETINOPATHY ARE AN URGENT UNMET NEED

Diabetic retinopathy (DR), a sight-threatening neurovasculopathy, is the leading cause of blindness in working-aged adults (Zhang et al., 2010; Hendrick et al., 2015). DR is characterized by pathologic vascular proliferation, oxidative damage, and inflammation within the retina (Tang and Kern, 2011; Antonetti et al., 2012; Klaassen et al., 2013). The progression of DR is highly correlated to the duration of diabetes (Fong et al., 2004). While restoring glycemic control and regulating other systemic factors are important for slowing DR development, limited therapeutic options are available once symptoms progress, and these are primarily aimed at treating late-stage disease (Fullerton et al., 2014; Do et al., 2015; Lee et al., 2015; Gardner and Sundstrom, 2017).

The number of DR patients is expected to increase over the coming decades. Currently, the only recommended treatments for advanced retinopathy are laser photocoagulation or anti-VEGF injections, but a substantial proportion of patients are resistant to these treatments. Laser photocoagulation can control pathological neovascularization, but it may lead to complications, such as impaired central vision, nocturnal diminution of vision, and blindness. The beneficial effects of anti-VEGF injections are usually transient, as the treatment does not promote tissue repair, and repeated injections increase the risk of intraocular infection. Furthermore, despite receiving anti-VEGF injections, a small proportion of patients with macular edema still show persistent disease (Lavine et al., 2017; Roy et al., 2017). Moreover, neither treatment targets early-stage disease. Another study examined the effect of candesartan, an angiotensin-II receptor antagonist, on patients with type 1 diabetes and found a moderate 18% reduction in incidence of retinopathy with no effect on the progression of existing retinopathy (Group and Chaturvedi, 2002). Therefore, new therapies to prevent retinal injury and enhance repair represent a critical unmet need.

INTERLEUKIN-6 TRANS-SIGNALING: A POTENTIAL THERAPEUTIC TARGET FOR DIABETIC RETINOPATHY

The pleiotropic cytokine, interleukin-6 (IL-6), is one of the major mediators of retinal vascular inflammation associated with DR (Shimizu et al., 2002; Funatsu et al., 2005; Mocan et al., 2006; Kawashima et al., 2007; Hou et al., 2008; Barnes et al., 2011; Koleva-Georgieva et al., 2011; Gustavsson et al., 2013; Koskela et al., 2013; Chen et al., 2016; Srividya et al., 2018; Valle et al., 2019). IL-6 signaling through its membrane-bound IL-6 receptor is known as "*classical signaling.*" Importantly, IL-6 signaling is also observed in cells

Edited and reviewed by:
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Specialty section:
This article was submitted to Redox Physiology, a section of the journal Frontiers in Physiology

Received: *31 March 2021*
Accepted: *12 April 2021*
Published: *19 May 2021*

Citation:
Sharma S (2021) Interleukin-6 Trans-signaling: A Pathway With Therapeutic Potential for Diabetic Retinopathy. Front. Physiol. 12:689429. doi: 10.3389/fphys.2021.689429

that do not express the membrane-bound IL-6 receptor through a soluble IL-6 receptor (sIL-6R), known as "*trans-signaling*" (Barnes et al., 2011; Rose-John, 2012). There is increasing evidence in the literature suggesting that IL-6 classical signaling is anti-inflammatory, whereas trans-signaling induces the pro-inflammatory effects of IL-6 (Rabe et al., 2008; Ebihara et al., 2011; Fisher et al., 2011; Scheller et al., 2011; Wei et al., 2013). Trans-signaling has also been reported to have stronger effects than classical signaling (Reeh et al., 2019).

Recent advances in the field have led to the development of several therapeutic interventions targeting IL-6 signaling pathways, including anti-IL6 antibodies: siltuximab, sirukumab, olokizumab, and clazakizumab; anti-IL6R antibodies: tocilizumab, sarilumab, satralizumab, and vobarilizumab; and selective inhibitors of IL-6 trans-signaling only: sgp130Fc (olamkicept). Anti-IL6 and anti-IL6R therapeutic strategies globally block IL-6 signaling, essentially targeting both classical and trans-signaling pathways. Tocilizumab, an IL-6 receptor-inhibiting monoclonal antibody, is useful in the treatment of various autoimmune and inflammatory conditions, notably rheumatoid arthritis (Ohsugi and Kishimoto, 2008). However, this treatment was associated with negative side effects, such as liver toxicity and increases in triacylglycerol and cholesterol levels (Kawashiri et al., 2011).

Long-term hyperglycemia-mediated oxidative stress and inflammation lead to blood-retinal barrier (BRB) dysfunction and increased vascular permeability, allowing extravasation of plasma proteins into the interstitium (Frey and Antonetti, 2011; Klaassen et al., 2013). This dysfunction leads to edema, deposition of hard exudates in the retina, microaneurysms, and retinal hemorrhage (Cheung et al., 2010; Cunha-Vaz et al., 2011; Eshaq et al., 2017). BRB breakdown and subsequent macular edema are the main causes of blindness in DR (Antonetti et al., 1999; Joussen et al., 2007; Gardner et al., 2009; Klaassen et al., 2013; Sugimoto et al., 2013; Kita et al., 2015; Lee et al., 2015). IL-6 plays a significant role in initiating BRB breakdown in DR (Mesquida et al., 2019; Valle et al., 2019). Studies have shown that IL-6 signaling decreases barrier function in retinal endothelial cells and increases vascular leakage through downregulating tight junction proteins (Yun et al., 2017; Jo et al., 2019). IL-6 trans-signaling causes oxidative stress, inflammation, and endothelial barrier disruption in human retinal endothelial cells (Valle et al., 2019). Further, in a mouse model of early DR, inhibition of IL-6 trans-signaling significantly reduced diabetes-induced oxidative damage at the systemic level and in the retina (Robinson et al., 2020).

IL-6 also plays an important role in localized immune responses by mediating the recruitment of circulating leukocytes, attachment to the endothelium, and migration through the vascular wall (Romano et al., 1997; Rojas et al., 2010; Ebihara et al., 2011). Arrest and firm adhesion of leukocytes occur by their binding to endothelial cells using intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). In diabetic patients, increased ICAM-1 expression in retinal vessels is correlated with an increase in migrating neutrophils (Noda et al., 2012). We previously found elevated levels of soluble ICAM-1 and VCAM-1 in patients with DR (Sharma et al., 2015) and increased ICAM-1 protein levels in human retinal endothelial cells after IL-6 trans-signaling activation (Valle et al., 2019). Numerous studies have demonstrated that IL-6 increases the expression of ICAM-1, VCAM-1, and selectins (Wung et al., 2005; Lin et al., 2013), but distinct roles of classical and trans-signaling have not been studied. Future studies delineating the relationship between IL-6 trans-signaling and leukocyte migration in the retinal vasculature will enhance our understanding of inflammation and BRB breakdown associated with DR.

MOLECULAR TOOLS FOR DELINEATING THE ROLES OF IL-6 CLASSICAL AND TRANS-SIGNALING

sgp130Fc

The soluble gp130 (sgp130) is a natural inhibitor of IL-6 trans-signaling (Wolf et al., 2016; Rose-John, 2017; Baran et al., 2018). The commercially available compound, sgp130Fc (soluble gp130Fc fused chimera), is an optimized fusion protein of the natural sgp130 and IgG1-Fc (Tenhumberg et al., 2008). sgp130Fc binds to IL-6 in complex with soluble IL-6R (IL-6/sIL-6R) and does not interfere with IL-6 alone or IL-6 bound to IL-6R on the cell surface. Therefore, sgp130Fc selectively inhibits IL-6 trans-signaling without disrupting IL-6 classical signaling via the membrane bound IL-6R. Compared to endogenous sgp130, sgp130Fc has been shown to possess 10 to 100 times greater ability for inhibiting IL-6 trans-signaling responses (Jostock et al., 2001). The use of this compound alongside existing global IL-6 inhibitors allows for a direct comparison of the therapeutic potential of global vs. selective trans-signaling inhibition.

Hyper IL-6

Hyper-IL-6 is a fusion protein using a flexible peptide linker between soluble IL-6R and IL-6 to connect both molecules. Therefore, instead of a mixture of IL-6 and soluble IL-6R, hyper IL-6 can be used to stimulate IL-6 trans-signaling in cells. Also, hyper IL-6 is ∼100× more potent than the combination of IL-6/sIL-6R (Fischer et al., 1997; Jostock et al., 2001; Drucker et al., 2010). This compound is particularly useful for studies involving cells that express the membrane-bound IL-6 receptor, as a mixture of IL-6 and soluble IL-6R could theoretically activate both classical and trans-signaling. Hyper IL-6 allows for selective activation of IL-6 trans-signaling without any classical signaling activation.

L-gp130

The transmission of the IL-6 signaling through the plasma membrane is mediated through glycoprotein 130 kDa (gp130). IL-6 receptor associates with the ubiquitously expressed protein gp130, initiating dimerization and intracellular signaling. L-gp130 is a designer protein in which the entire extracellular portion of gp130 is replaced by the leucine zipper of the Jun protein for constitutive dimerization and activation. Thus, L-gp130 protein can be used for permanent gp130 activation to mimic constitutive IL-6 signaling in cells (Stuhlmann-Laeisz et al., 2006).

Transgenic Mice Overexpressing sgp130Fc

Transgenic mice that constitutively overexpress sgp130Fc are valuable resources to selectively block IL-6 trans-signaling *in vivo* (Rabe et al., 2008). Two types of transgenic mice are available for either central or peripheral expression of sgp130Fc. Peripheral sgp130Fc transgenic mice express sgp130Fc in the liver under the control of the phophoenol pyruvate-carboxykine (PEPCK) promotor for systemic release into the circulatory system (Rabe et al., 2008; Kraakman et al., 2015). The central sgp130Fc transgenic strain allows for inhibition of IL-6 trans-signaling in the central nervous system through sgp130Fc expression under control of a glial fibrillary acidic protein (GFAP) promotor (Campbell et al., 2014). Functionally, these models mimic intravenous (peripheral) or intravitreal (central) drug delivery, two common methods used in the treatment of ocular diseases.

CONCLUDING REMARKS

Increasing evidence suggests that the IL-6 pathway plays a prominent role in the pathogenesis of DR. The complex IL-6 receptor system allows for multiple signaling modalities, including classical signaling and trans-signaling. Classical signaling is critical for the regenerative or anti-inflammatory activities of IL-6, while recent studies have demonstrated that IL-6 trans-signaling is primarily pro-inflammatory. In DR, IL-6 trans-signaling mediates barrier disruption in retinal endothelial cells, and blockade of this pathway maintained normal endothelial barrier function. Selective inhibition of IL-6 trans-signaling with sgp130Fc also suppressed ocular inflammation and oxidative stress in a mouse model of DR. These findings indicate that a pathway primarily driven by IL-6 + soluble IL-6R contributes to vascular inflammation in the diabetic retina. Therefore, inhibiting only the trans-signaling pathway of IL-6 will likely be therapeutically superior to a complete IL-6 blockade, because important physiologic functions of IL-6 classical signaling will remain intact. An emerging challenge is identifying means of targeting this inflammatory pathway, as well as determining which DR patients may benefit most from therapies blocking IL-6 trans-signaling. The selective inhibition of IL-6 trans-signaling using the sgp130Fc fusion protein is in clinical trials for the treatment of several inflammatory diseases (Rose-John, 2017) and may be repurposed in the future as an excellent target for DR therapy.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

FUNDING

This study was supported by the National Institutes of Health, National Eye Institute (Bethesda, MD, USA) grant # R01-EY026936 awarded to SS.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

SPECIALTY GRAND CHALLENGE
published: 30 April 2021
doi: 10.3389/fphys.2021.682467

Looking Back to the Future of Mitochondrial Research

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Keywords: mitochondria, ATP synthase, channels, pathophysiology, hypoxia

῾Ηθος ἀνθρώπῳ δαίμων–*ethos anthropoi daimon*—is a famous aphorism of the Greek philosopher Heraclitus (544–483 BC). While its deeper meaning is probably more complex, the conventional translation is "a human being's character is his/her fate." When I was asked by George Billman to contribute my thoughts on the future of mitochondrial research it occurred to me that perhaps I could try to foretell the fate of mitochondrial research from its character, i.e., from the key themes from which the discipline developed. I will limit this brief comment to a few topics that also reflect my own interests, and that should not be considered even an attempt to be exhaustive. In the twentieth century the key issue in Bioenergetics (hence in mitochondrial research) has been the mechanism of energy conservation. The turning point was the proposal and then the demonstration of Peter Mitchell's chemiosmotic hypothesis, i.e., that in mitochondria the basic events are the coupling of aerobic electron transfer to H^+ pumping, the formation of the H^+ electrochemical gradient and its harnessing by the ATP synthase (Mitchell, 1966), reprinted in Mitchell (2011). It is remarkable that the most recent advances in structural biology and superresolution microscopy, which are removing hurdles and moving the boundaries of Science beyond imagination, have confirmed the basic tenets of chemiosmotic principles in amazing detail.

ATP SYNTHASE

One example of how structural biology complemented physiology is the solution to the question of the H^+/ATP stoichiometry, which had been the matter of considerable discussion (Brand and Lehninger, 1977; Sholtz et al., 1983). The demonstration that different organisms possess c rings (the rotating barrel powered by H^+ flux) with a number of subunits varying between a minimum of 8 and a maximum of 17, and the fact that one full rotation cycle generates 3 ATP molecules (Boyer, 1997) has both defined the precise stoichiometry for each type of c ring (between 2.67 and 5.67 H^+/ATP) and explained the apparent variability in the stoichiometry itself, see Nirody et al. (2020) for a review. While the basic structure and catalytic mechanism of ATP synthases is highly conserved across species, what is most puzzling is the existence of profound differences in the non-catalytic parts of the enzyme, which evolved to include a numer of subunits that were indeed defined "supernumerary" (Vaillier et al., 1999) because they are not essential for the catalytic activity. These subunits are involved in the process of dimerization and of membrane bending that contributes to generate the inner membrane cristae, which are then stabilized by the lateral association of dimers (Paumard et al., 2002; Dudkina et al., 2006; Jiko et al., 2015; Kühlbrandt, 2019; Spikes et al., 2021). And yet, clear species-specific differences exist in primary structure of these subunits, suggesting that they may serve additional function(s) that have not been fully discovered yet (Kühlbrandt, 2019). I think that research on the physiological function(s) of "supernumerary" subunits of ATP synthases (which may include the controversial formation of the high-conductance permeability transition pore) (Giorgio et al., 2013; Alavian et al., 2014; He et al., 2017a,b; Carroll et al., 2019; Mnatsakanyan et al., 2019; Urbani et al., 2019; Pinke et al., 2020) will be a very fruitful field of investigation in the years to come.

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Mitochondrial Research, a section of the journal Frontiers in Physiology

Received: *18 March 2021*
Accepted: *12 April 2021*
Published: *30 April 2021*

Citation:
Bernardi P (2021) Looking Back to the Future of Mitochondrial Research. Front. Physiol. 12:682467. doi: 10.3389/fphys.2021.682467

A SUPERCOMPLEX MATTER

High-resolution definition of individual mitochondrial respiratory complexes, which begun over 20 years ago, has also yielded key information on the mechanisms of proton translocation coupled to electron transfer (Tsukihara et al., 1996; Xia et al., 1997; Michel et al., 1998; Baradaran et al., 2013; Zickermann et al., 2015; Kampjut and Sazanov, 2020). How electrons are transferred *between* respiratory complexes and whether this requires stable interactions is a question of great relevance, that to the best of my knowledge was clearly posed and addressed with inhibitor titration studies by Stoner (1984). The existence of respiratory supercomplexes made by associations of complexes I, III, and IV with defined stoichiometries in the native membrane is now established, and the role of supercomplexes in pathophysiology is increasingly appreciated (Schägger and Pfeiffer, 2000, 2001; Bianchi et al., 2004; Acín-Pérez et al., 2008; Dudkina et al., 2011; Lapuente-Brun et al., 2013; Letts et al., 2016; Milenkovic et al., 2017; Rathore et al., 2019; Berndtsson et al., 2020; Protasoni et al., 2020). An important question is the role of the supercomplex assembly factor SCAF1, which has been shown to promote supercomplex formation between CIII and IV (III_2IV) and contribute to "branching" of the respiratory chain (Calvo et al., 2020). This topic is generating a very lively discussion that is likely to continue in the future (Mourier et al., 2014; Enríquez, 2016; Milenkovic et al., 2017; Lobo-Jarne et al., 2018; García-Poyatos et al., 2020; Protasoni et al., 2020; Fernández-Vizarra et al., 2021).

DE(LOCALIZED) GRADIENTS?

Another interesting question is whether H^+ pumping (together with the low permeability of the inner membrane) can generate a *delocalized* electric field rapidly spreading to the whole network (Amchenkova et al., 1988), or rather mitochondria should be seen as a mosaic of *localized* coupling units where the H^+ pumping complexes and ATP synthases are closely spaced to make individual functional units without the need for lateral diffusion of charges (Yaguzhinsky et al., 2006), two hypotheses that may actually not be mutually exclusive (Westerhoff et al., 1986). Recent work using high resolution microscopy has demonstrated that both have merit and that, to some extent, the issue may be a matter of definition (i.e., what is meant by "short" and "long" range) and possibly of anatomy. Long range diffusion may predominate in tissues were mitochondria are mostly in the form of tubular, continuous structures (like in muscle) (Glancy et al., 2015) while local coupling (with formation of individual disc-shaped crista structures) may prevail where (or when) mitochondria are in the form of individual organelles (Wolf et al., 2019). This area of research has also made great progress on the fusion-fission events that regulate mitochondrial morphology and function (Giacomello et al., 2020). I think that superresolution microscopy together with the genetic manipulation of determinants of mitochondrial morphology will allow further definition of subcellular electrical events that bear both on mitochondrial function and on the shaping of localized ion gradients.

MITOCHONDRIAL DYNAMICS

Mitochondrial dynamics is a topic of enormous interest and of great additional potential in spite of the major progress made in recent years. The pioneering work of Jürgen Bereiter-Hahn provided a detailed description of mitochondrial motion and of fusion-fission events *in vivo* (Bereiter-Hahn, 1990; Bereiter-Hahn and Voth, 1994). The molecular basis for mitochondrial dynamics is being unraveled at a steady pace, and is revealing the delicate balance between proteins that favor mitochondrial fusion and those that promote mitochondrial fission, their relationships with the cell cycle and with mitochondrial responses to pathophysiological perturbations (which depend on the cell type as much as on the stimulus), their role in cell survival and death, and their relationship to proteins that determine the maintenance of mitochondrial ultrastructure and its close interactions with the endoplasmic reticulum (Osteryoung and Nunnari, 2003; Mishra and Chan, 2016; Eisner et al., 2018; Giacomello et al., 2020).

CATION CHANNELS

When thinking of mitochondria and subcellular, localized ion gradients one obviously thinks of Ca^{2+} (Rizzuto et al., 1993) and this takes us to one of the paradoxes that accompanied the progressive acceptance of the chemiosmotic hypothesis. The existence of a proton electrochemical gradient as the energetic intermediate between respiration and ATP synthesis poses some constraints on membrane permeability to cations. Indeed, it was noted that equilibrium distribution of K^+ and Ca^{2+} across a membrane maintaining an electrical potential difference of 180 mV (negative inside) would have meant matrix concentrations of about 150 M for K^+ and 1 M for Ca^{2+}, see Bernardi (1999) for a detailed review. Mitchell was aware of this problem and conceived two complementary postulates to solve it. The first (3rd postulate of chemiosmosis) is that the inner membrane possesses electroneutral H^+-cation exchangers allowing extrusion of cations entering the matrix down their electrochemical gradient. Operation of the exchangers (Mitchell and Moyle, 1969; Garlid, 1978, 1979) prevents the otherwise inevitable accumulation of cations that would lead to swelling and osmotic lysis of the organelle. The quest for the K^+-H^+ exchanger is still under way, although the LETM1 protein is clearly involved in mitochondrial cation homeostasis through modulation of the K^+-H^+ exchange process (Nowikovsky et al., 2004, 2007) and possibly also of electroneutral Ca^{2+}-H^+ exchange (Tsai et al., 2014), an issue that is still the matter of discussion (De Marchi et al., 2014; Nowikovsky and Bernardi, 2014), see Austin and Nowikovsky (2019) for a recent review. The second (4th postulate of chemiosmosis) is that the inner membrane has a low permeability to protons and to anions and cations generally (Mitchell, 1966, 2011). The latter point was almost universally (and as it turns out, erroneously) taken to mean that mitochondria could not possess channels for cations, a point that pervaded the literature well until the turn of last century (Garlid et al., 1989). This state of affairs considerably delayed the discovery of the mitochondrial Ca^{2+} uniporter (MCU) and its regulatory subunits (Perocchi

et al., 2010; Baughman et al., 2011; De Stefani et al., 2011; Mallilankaraman et al., 2012; Sancak et al., 2013; Kamer and Mootha, 2014; Mammucari et al., 2016) and the assessment of their role in disease (Logan et al., 2014; Debattisti et al., 2019); and of mitochondrial K^+ channels (Inoue et al., 1991; Szewczyk et al., 2006; Szabó and Zoratti, 2014; Paggio et al., 2019). The great wave coming from these areas of research is unlikely to subside, and will translate in more breakthroughs on how mitochondrial participate and contribute to the shaping of intracellular ion gradients.

INNER MEMBRANE PERMEABILITY AND PATHOPHYSIOLOGY

As the chemiosmotic hypothesis became consolidated, a set of early observations on the Ca^{2+}-dependent permeability increase to ions and solutes through "permeability defects" with a pore radius of 14 Å (Massari and Azzone, 1972) became widely interpreted as an *in vitro* artifact of little relevance to mitochondrial physiology, see Bernardi et al. (2006) for a specific review. Only a few Authors interpreted the increased permeability (defined permeability transition, PT, by Haworth and Hunter) as a potentially regulated event serving a role in pathophysiology (Haworth and Hunter, 1979; Hunter and Haworth, 1979a,b; Pfeiffer et al., 1979; Crompton et al., 1987), possibly as a regulated pathway for Ca^{2+} release (Bernardi and Petronilli, 1996), which is consistent with a number of observations (Carraro et al., 2020). While today there is a general agreement that the PT is mediated by opening of a channel, its molecular identity is the matter of discussion. The latest results suggest that the PT can be mediated by a Ca^{2+}-dependent conformational change of both the adenine nucleotide translocator (ANT) and the ATP synthase, through mechanisms that still need to be defined, see Carraro et al. (2020) for a discussion. The PT has been shown to play a role in necrotic cell death in a set of studies (Duchen et al., 1993; Imberti et al., 1993; Pastorino et al., 1993) that were greatly helped by the demonstration that the PT is inhibited by cyclosporin A (Fournier et al., 1987; Crompton et al., 1988; Broekemeier et al., 1989) through the matrix protein cyclophilin D (Halestrap and Davidson, 1990; Nicolli et al., 1996). The PT was then shown to play a role in apoptosis as well (Marchetti et al., 1996). Together with the discovery that cytochrome c release from the intermembrane space triggers the mitochondrial pathway of apoptosis through activation of procaspase 9 (Liu et al., 1996), these studies opened a new season in mitochondrial research that is lasting to this day for its major implications in the pathogenesis of both degenerative diseases and cancer. Selective cytochome c release can be achieved by Bax/Bak-dependent permeabilization of the outer mitochondrial membrane following insertion of tBid generated by activation of caspase 8 (Wei et al., 2000) in a process that is substantially opposed by the antiapoptotic protein Bcl-2 (Susin et al., 1996; Yang et al., 1997). Release of cytochrome c can also be a consequence of PTP-dependent swelling (Petronilli et al., 1994) and/or cristae remodeling (Scorrano et al., 2002), and there is an intriguing promoting effect of Bax/Bak (Karch et al., 2013) and an inhibitory effect of Bcl-2 on onset of the PT (Susin et al., 1996). The latter is contrasted by Bcl-2 small molecule interactors (Milanesi et al., 2006) able to reactivate the mitochondrial death program (Oltersdorf et al., 2005) and these regulatory events extend to a variety of Bcl-2 family members (Singh et al., 2019). In a striking therapeutic development, the Bcl-2 ligand ABT-199 (venetoclax) has been introduced in the treatment of a variety of hematologic malignancies (Souers et al., 2013; de Ridder et al., 2021). It should also be mentioned that mitochondria play a key role in degenerative diseases, particularly muscular dystrophies and neurodegenerative conditions ranging from Parkinson's to Alzheimer diseases, amyotrophic lateral sclerosis, multiple sclerosis; and in organ ischemia-reperfusion injury. The mechanisms and targets, which include the PT, are so many that I will not even try to list them, but I would like to mention early work that anticipated these modern developments of mitochondrial pathophysiology (Hunter and Ford, 1955; Kasbekar and Sreenivasan, 1956; Hoch, 1962; Luft et al., 1962; Wollenberger et al., 1963; van Wijngaardeen et al., 1967; Sternlieb, 1968; Jennings et al., 1969; Fleckenstein et al., 1974; Wrogemann and Pena, 1976; Singer et al., 1987), see Bernardi et al. (2015) for relevant literature.

ADENINE NUCLEOTIDE TRANSLOCATOR AND UNCOUPLING PROTEINS

Another historical area of research where breakthroughs are being made is that of nucleotide transport via the ANT. It had long been proposed that the overall exchange of ADP for ATP was mediated by a single substrate-binding site alternately accessible from either side of the membrane (Klingenberg, 1979; Ruprecht et al., 2014). The most recent structures fully confirm this single-pore gating mechanism, whereby in energized mitochondria the nucleotide exchange reaction is mediated by unidirectional uptake of ADP and efflux of ATP "taking turns" on the carrier (Ruprecht et al., 2019). Many issues still await an answer, however. It has recently been shown that in the presence of arachidonic, palmitic or lauric acid the ANT can also transfer H^+ in mitochondria that do not express uncoupling protein 1 (UCP1) (Bertholet et al., 2019), the bona fide H^+ channel that mediates non-shivering thermogenesis in brown fat (Nicholls, 1976; Rafael and Heldt, 1976). The existence of ANT-mediated H^+ currents detected in patch-clamp experiments (Bertholet et al., 2019) supports the earlier suggestion that the ANT mediates a sizeable fraction of the "H^+ leaks" responsible for basal respiration (Andreyev et al., 1988; Brustovetsky and Klingenberg, 1994). ANT and UCP1 are closely related proteins and both require long-chain fatty acids for H^+ translocation and yet the molecular mechanisms appears to differ, as only in UCP1 the fatty acid anion participates in the actual mechanism of H^+ transport (Fedorenko et al., 2012) while it plays a cofactor role in ANT (Bertholet et al., 2019), see Bernardi (2019) for a summary. It will be interesting to test whether other members of the SLC25 superfamily of mitochondrial solute carriers (Palmieri and Monné, 2016) can mediate the occurrence of H^+ leaks. An additional open question about the ANT is how it can be

transformed by Ca^{2+} in a high-conductance channel stimulated by cyclophilin D with an effect prevented by the cognate inhibitor of the latter cyclosporin A (Brustovetsky and Klingenberg, 1996; Brustovetsky et al., 2002), see Carraro et al. (2020) for a recent discussion.

MITOCHONDRIAL DNA

Mitochondria possess their own DNA and translation machinery. Diseases of mtDNA have first been described not so long ago (Wallace et al., 1988), and a new frontier is the manipulation of mtDNA, which holds great promise for a future correction of mtDNA diseases (Gammage et al., 2018) and possibly to treat cancer (Bonekamp et al., 2020). It is remarkable that only 13 out of the roughly 1,100 proteins found in mitochondria are encoded by mtDNA (Rath et al., 2020). During evolution mtDNA has retained only a core set of genes of the respiratory chain and F-ATP synthase, possibly to permit rapid adaptation to changing environments (Wallace, 2007). How mitochondrial and nuclear genomes integrate in mitochondrial biogenesis remains a fascinating topic (Becker et al., 2019) as is the somewhat specular issue of how cells exploit mitochondrial "diversity" by releasing into the circulation mitochondrial damage-associated molecular patterns (including mtDNA) to engage toll-like receptors and innate immune pathways (Zhang et al., 2010; Shintani et al., 2014; Rodríguez-Nuevo et al., 2018) and activate inflammation (Zhou et al., 2011; Oka et al., 2012; Zhong et al., 2018). This is strikingly similar to the effect of microbial pathogen-associated molecular patterns and provides an exciting link to STING, which regulates the type I interferon response (Sliter et al., 2018). The mechanism for mtDNA release is an interesting issue on its own, because it could be a regulated process mediated by the permeability transition pore (Yu et al., 2020) rather than the unspecific result of cell damage, an issue that will certainly attract more attention.

AN UNEXPECTED TWIST ON HYPOXIA

The discovery that mitochondria are involved in the HIF-mediated response to hypoxia (Samanta and Semenza, 2018) through succinate-dependent stabilization of HIF-1α (Selak et al., 2005) and modulation of expression of cytochrome oxidase subunits (Fukuda et al., 2007) was a turning point for our understanding of metabolic adaptation of tumors, first proposed as a causative event in cancer by Otto Warburg (Warburg et al., 1927; Warburg, 1956). A further mechanistic link was provided by the demonstration that TRAP1, a protein targeted to mitochondria in many tumors, inhibits succinate dehydrogenase and leads to succinate accumulation, stabilizing HIF-1α under normoxic conditions and thus making tumor cells ready to resist the impending onset of hypoxia (Sciacovelli et al., 2013). The unexpected twist is that hypoxia has a *beneficial* effect in disorders of the respiratory chain through activation of an endogenous program that allows adaptation. Chronic hypoxia led to a marked improvement in survival and vital parameters in a mouse model of Leigh syndrome, an effect that could not be explained by activation of the HIF transcriptional program (Jain et al., 2016). Rather, mice underwent an age-dependent decline in overall oxygen consumption with brain hyperoxia, which was normalized by hypoxic breathing, carbon monoxide or severe anemia with matching reversal of the neurological disease (Jain et al., 2019). These exciting new results suggest that unused oxygen rather than hypoxia itself may be the culprit, and open up new perspectives to normalize brain tissue hyperoxia (Jain et al., 2019). Genome-wide CRISPR screens at low oxygen tension have now identified genes with relative fitness defects in high or low oxygen, and most of these did not have an obvious connection to HIF (Jain et al., 2020). Remarkably, knockouts of mitochondrial pathways that are presumed to be essential, including complex I, grew relatively well at low oxygen (Jain et al., 2020). This approach is leading to the discovery of hundreds of genes linked to oxygen homeostasis, and there is more. Hypoxia has recently been shown to induce matrix acidification with release of Ca^{2+} from calcium phosphate precipitates, increased free [Ca^{2+}] and matrix influx of Na^+ on the Na^+/Ca^{2+} exchanger (Hernansanz-Agustín et al., 2020). Na^+ interaction with phospholipids then reduced inner membrane fluidity, selectively decreasing mobility of free ubiquinone between complex II and III but not inside supercomplexes, thus leading to increased superoxide production at complex III, a novel control mechanism of redox signaling that may have profound consequences for cellular metabolism (Hernansanz-Agustín et al., 2020).

INTRAORGANELLE BUFFERING

Another topic that I find particularly fascinating is intramitochondrial communication between the two "arms" of oxidative phosphorylation, i.e., the respiratory chain and the ATP synthase. Respiratory complex III is assembled from a core containing cytochrome b (the only component encoded by mtDNA) and subunits Qcr7 and Qcr8, followed by the incorporation of all other subunits (Smith et al., 2012). As is the case with other respiratory complexes, specific proteins are required for the assembly of complex III including Bcs1 (Nobrega et al., 1992), an assembly factor that is the most frequent target of mutations in human complex III-related diseases. Extragenic compensatory mutations of yeast *bcs1* have been identified that preferentially target the ATP synthase complex, leading to selective decrease of its ATP hydrolytic activity with substantial preservation of ATP synthesis (Ostojic et al., 2013). Thus, the bioenergetics consequences of respiratory impairment appear to be limited by minimizing the hydrolysis of ATP. These results have recently been extended in a thorough study of the Mootha laboratory, who have found that the cellular defects derived from chemical inhibition of complex V with oligomycin are suppressed by loss of complex I activity induced by both genetic and pharmacological means (To et al., 2019). This is a striking example of "intra-organelle" buffering that was also seen for a variety of other mitochondrial inhibitors, suggesting that certain forms of mitochondrial dysfunction may be buffered with "second site" inhibition within the organelle (To et al., 2019). Consistent with the existence of a regulatory feedback between biogenesis of respiratory complexes and of

the ATP synthase, ablation of specific subunits of ATP synthase (that largely prevented its assembly) caused a striking decrease of electron transfer chain complexes, with reduction of respiration to negligible rates (He et al., 2017a,b; Carroll et al., 2019).

NOT ALL COULD BE PREDICTED

The more I tried to cover new perspectives that are rooted in the history of mitochondrial research, the more I realized that my selective account was inevitably leaving out a number exciting developments. I will mention the relationships of autophagy with mitochondrial fission-fusion events (Twig et al., 2008; Lazarou et al., 2015; Dorn, 2016); the role of mitochondria in the antiviral response (Kozaki et al., 2017), in the growth of intracellular parasites (Pernas et al., 2018), in antigen presentation (Matheoud et al., 2016), in T cell function (Okoye et al., 2015; Weinberg et al., 2019) and dysfunction (Desdin-Mico et al., 2020), in metabolic reprogramming of macrophages (Mills et al., 2016; Acín-Pérez et al., 2020), in angiogenesis (Herkenne et al., 2020), in systemic stress response mediated by FGF21 (Forsstrom et al., 2019), in non-alcoholic steatohepatitis, where downregulation of mitochondrial circular RNA prevents inhibition of the permeability transition pore by the SCAR protein (Zhao et al., 2020); recent advances on the mechanism of germline selection of human mtDNA (Wei et al., 2019); and the most unexpected finding that the protein product of the *ARHGAP11B* gene, which plays an essential role in development of the human neocortex (Heide et al., 2020), localizes to mitochondria to inhibit the permeability transition pore (Namba et al., 2020). It is reassuring, indeed, that not all could be predicted.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

FUNDING

Research in my laboratory was supported by Associazione Italiana Ricerca sul Cancro (IG17067), Fondation Leducq (16CVD04), the Italian Ministry for University and Research (2017LHFW42) and Telethon (GGP17092).

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

PERSPECTIVE
published: 29 July 2020
doi: 10.3389/fphys.2020.00588

Early Career Scientists' Guide to the Red Blood Cell – Don't Panic!

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Why should we take interest in studying red blood cells? This mini review attempts to answer this question and highlights the problems that authors find most appealing in this dynamic research area. It addresses the early career scientists who are just starting their independent journey and facing tough times. Despite unlimited access to information, the exponential development of computational and intellectual powers, and the seemingly endless possibilities open to talented and ambitious early career researchers, they soon realize that the pressure of imminent competition for financial support is hard. They have to hit deadlines, produce data, publish, report, teach, manage, lead groups, and remain loving family members at the same time. Are these countless hardships worth it? We think they are. Despite centuries of research, red blood cells remain a mysterious and fascinating study objects. These cells bring together experts within the family of the European Red Cell Society and beyond. We all share our joy for the unknown and excitement in understanding how red cells function and what they tell us about the microenvironments and macroenvironments they live in. This review is an invitation to our colleagues to join us on our quest.

Keywords: red blood cell, senescence, Clearance, adaptation, Comparative, function, morphology

OPEN ACCESS

Edited by:
Geoffrey A. Head, Baker Heart and Diabetes Institute, Australia

Reviewed by:
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Specialty section:
This article was submitted to Red Blood Cell Physiology, a section of the journal Frontiers in Physiology

Received: *02 January 2020*
Accepted: *11 May 2020*
Published: *29 July 2020*

Citation:
Bogdanova A and Kaestner L (2020) Early Career Scientists' Guide to the Red Blood Cell – Don't Panic!. Front. Physiol. 11:588. doi: 10.3389/fphys.2020.00588

WITH INSPIRATION AND IN MEMORY OF DOUGLAS ADAMS AND HIS ULTIMATE HITCHHIKER'S GUIDE TO THE GALAXY (Adams, 1996)

Red blood cells have power over us, no doubt. Making up over 50% of our cells (2×10^{13} cells), these cells provide us with energy to live, think, and create (Bianconi et al., 2013; Lew and Tiffert, 2017). Each day, we lose 1.7×10^{11} cells and make the same number anew (Lew and Tiffert, 2017). However, the deeper we delve into the red blood cell universe, the humbler we feel, as we still have no ultimate answer to "the Question of Life, the Universe, and Everything."

Are all the 1.7×10^{11} cells we produced today the same? Do they differ from the cells we made yesterday when we went hiking in the mountains or were swimming in the lake? How do the heat waves associated with climate change affect these cells? How does microgravity affect them when, e.g., hitchhiking through the galaxy? How do the cells change as we get older and older with the increasing life expectancy?

Do the red cells produced today pass away on the same day and from the same cause? For humans, the causes of death and lifespan of individual cells seem to be somewhat random (Kaestner and Minetti, 2017), and the investigations to be done are complex, as we cannot trace

the life cycle of each individual cell back as detectives do. We do not know exactly what forces red blood cells to die and how they exactly cease to exist. Some of us acknowledge oxidation-induced clustering of band 3 proteins in the membrane as a signal that tags cells with antibodies against these clusters as "labeled for removal" (Lutz, 2012; Lutz and Bogdanova, 2013). Others describe red cell death as an "apoptosis-like" process and call it eryptosis (Lang et al., 2006, 2008). The "Nomenclature Committee on Cell Death," specializing in terminology, warns us for translation of the process as well as the word "eryptosis" to the way the enucleated cells pass away (Galluzzi et al., 2018). Is there one or more than one way to die? This question remains unclear and must still be resolved.

What do we need to learn about red blood cells? As in Adams' novels, experts and early career scientists witness dynamic developments in the field that leave us both excited and thrilled. We seem to know much about the major function of red blood cells, which is gas transport, but there is much more to what these cells are doing. This makes it difficult to limit the number of ultimate questions to just one (**Figure 1**).

There are more than 100 years of evidence for the active participation of red blood cells in blood coagulation (Duke, 1983; Andrews and Low, 1999; Steffen et al., 2011; Byrnes and Wolberg, 2017). This concept, however, did not mature enough to enter the textbooks. How many million years will it take?

There are some indications that red blood cells may sense the changes in plasma levels of hormones, such as insulin (Pedersen et al., 1982; Zhang et al., 2015), catecholamines (Hasan et al., 2017) and cortisol (Farese and Plager, 1962), sex hormones (Koefoed and Brahm, 1994), and erythropoietin (Trial et al., 2001; Mihov et al., 2009). If so, what happens to the cells as hormones interact with the receptors on the red blood cell membrane or cytosolic components?

Red blood cells are famous because they are widely used as a perfect cell model for studying cell membranes (Agre and Parker, 1989; Bernhardt and Ellory, 2003; Yawata, 2003). Earlier, all cells looked the same to the observers, and their properties were studied "en masse" e.g. using radioactive isotopes, rubbing cells between two plates to examine their viscosity and deformability, by inflating or deflating them, and by applying all possible approaches to whole blood samples. Mean volume and hemoglobin content values were assigned to them. Only desperate experts, such as Marcel Bessis, were photographing red blood cells for their beauty and turning their appearance into art by means of scanning electron microscopy (Bessis, 1974). If we want to study cells of similar densities and, eventually, ages, we may apply centrifugal force to produce fractions of such cells (**Figure 2**). Everyone that has once done such an experiment appreciates that each red cell has a certain density and joins one, but not the other group of cells of certain density, producing a striped pattern in centrifuged samples. Why certain densities are favored and others avoided is a question that needs answering. Our current understanding of red blood cell shapes includes their individual appearance and properties, their dynamic shape transitions, and their "shape memory" (Fischer, 2004; Tomaiuolo, 2014; Lanotte et al., 2016; Cordasco and Bagchi, 2017; Kihm et al., 2018). These studies make use of cellular and molecular biophysics, sophisticated *in vivo* imaging techniques for microfluidic channels and even blood vessels, and a great deal of artificial intelligence – even coming close to "Deep Thought," the supercomputer in Adams'

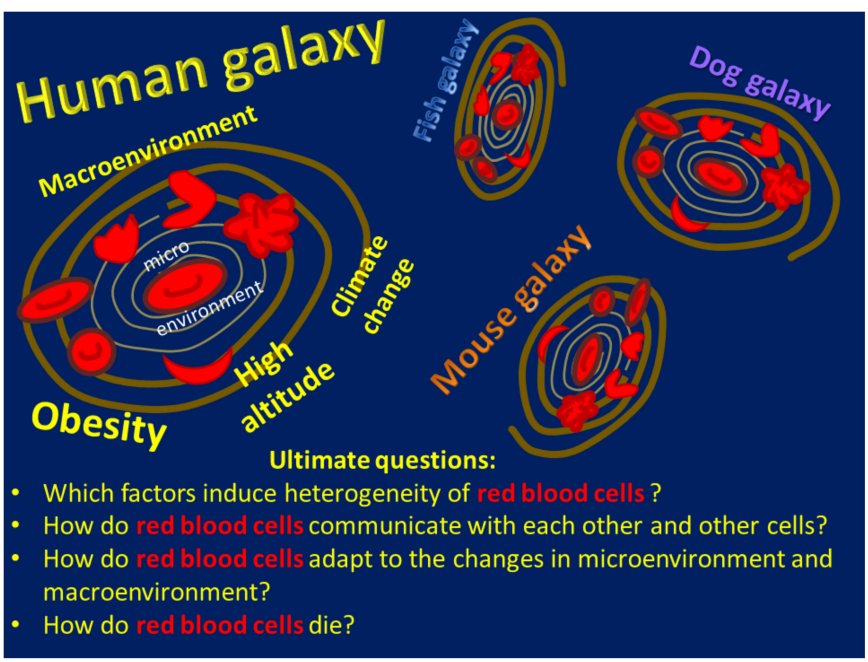

FIGURE 1 | A schematic of the Red Blood Cell Universe as the authors see it, including a list of ultimate questions.

FIGURE 2 | Red blood cells may have different densities, as shown on this continuous Percoll density gradient, but some densities are favored, and others are mysteriously abandoned.

story – but there is one crucial difference: patients do not have a million years to wait for the answer.

Most red blood cell researchers are deeply devoted to developing novel tools that may reveal so far unnoticed sides of cells we all like to study. Benefits are include protein profiling of red blood cells using omics approaches (Nemkov et al., 2018). Technologies use stem cells for production and manipulation of red blood cells (Hansen et al., 2019). We may follow red blood cells running through the blood vessels of living hosts (Hertz et al., 2019; Slovinski et al., 2019) and model and evaluate responses of red blood cells to mechanical shear forces – the ones they are exposed to in our microvasculature (Lizarralde Iragorri et al., 2018; Moura et al., 2019). We can detect electric currents that ions mediate passing through red blood cell membranes in hundreds of individual cells at the same time (Rotordam et al., 2019). As progress in research is fast, sometimes careful examination of the possible pitfalls and sources of artifacts is required (Minetti et al., 2013).

There is even more to explore in the universe of pathophysiology. For some patients, we do not have an answer as to what causes a red blood cell defect and only know the symptoms. Even when the molecular cause of the disease is clear, the links between the defective protein (a mismatch in amino acid composition or a dysregulated production program) and disease severity are often unknown. This is the case for sickle cell disease, hereditary spherocytosis, and Gardos channelopathy, just to name a few examples. Some patients get scientists involved into a thrilling quest for the actual cause of disease, those that were identified by hematologists as carriers of "idiopathic hemolytic anemia." New tools are currently in development that will enable the diagnosis of "newly identified" diseases (Kaestner and Bianchi, 2020).

Even more mysterious cases are described when defects in red blood cells come along with neurodegenerative symptoms. One such disease was named "acanthocytosis" (from the Greek word "acantha," meaning "thorn") because of the spiked thorny appearance of red blood cells (De Franceschi et al., 2014; Adjobo-Hermans et al., 2015). In fact, neurons and erythroid progenitor cells in the bone marrow were recently shown to share common gene regulatory pathways defining their fate and properties (Kinney et al., 2019).

The heart and blood also have much in common (Kaestner, 2013). The mortality of patients with myocardial infarction (acute coronary syndrome) and those undergoing valve replacement surgery may be predicted based on the degree of variance in red blood cell shapes and sizes (Ghaffari et al., 2016; Duchnowski et al., 2017; Abrahan et al., 2018). Furthermore, red blood cells were recently shown to function as actors, not passive witnesses, in cardiovascular diseases, contributing to the regulation of redox state and vascular tone and activating protective or disruptive signaling cascades in the myocardium and blood vessels (Pernow et al., 2019). Can red blood cells be regarded as deputies for other organs of our body, such as the brain and the heart?

One more exciting and rapidly developing area aims at revolutionizing blood donations and transfusions. Instead of relying on people readily offering their blood for the others to use, researchers are producing, so far in very small amounts, red blood cells of the type needed for each individual patient in a test tube (Shah et al., 2014; Hawksworth et al., 2018). However, it will take some time until cell culture can upscale to provide enough red blood cells for transfusion. Therefore, before this happens, we still have to rely on blood donations and do our best to improve the red blood cell storage conditions (D'Alessandro and Seghatchian, 2017) and to manage and reduce damage of cells during lesions (Yoshida et al., 2019). Furthermore, each patient may decide in the future to use his/her own red blood cells as transport containers to deliver toxic drugs to the location in the body where they are supposed to act without poisoning the host (Villa et al., 2016; Sun et al., 2017). Nature itself has chosen to modify components of red blood cells to protect hosts from *Plasmodium* infection causing a deadly disease that claimed over 400 000 lives in 2018 alone, malaria (Weatherall, 2008; Timmann et al., 2012; Malaria Genomic Epidemiology et al., 2015). This evolutionary selection has taken ages to occur and may now be of use to the development of protective strategies for the human population, as the spread of Plasmodium further to the north will follow the increase in atmospheric temperatures.

The universe of red blood cells spreads far beyond the cells that function in *Homo sapiens*. In agreement with the Hitchhiker's Guide, we have learned much about RBCs in the true rulers of the Earth, mice. These furry fellows give us a chance to study the mechanisms of diseases and to design new therapies for mice and humans. Our knowledge of the red blood cells in other species, including our pets and other tamed and wild, warm- and cold-blooded creatures that attend veterinary clinics from time to time, is rather fragmentary and requires more attention (**Figure 1**).

The ultimate "Answer to the Ultimate Question of Life, the Universe, and Everything" may only be given as we keep working and using our brains along with artificial intelligence. The next edition of "The Guide to Red Blood Cells" is on the way, and the motto for the early career scientists in the area stands as stated by Adams: "Don't Panic."

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 09 November 2020
doi: 10.3389/fphys.2020.542466

Avian Physiology: Are Birds Simply Feathered Mammals?

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There are marked differences between the physiology of birds and mammals. These reflect the evolutionary distance between the two classes with the last common ancestor estimated as existing 318 million years ago. There are analogous organ systems in birds and mammals. However, marked differences exist. For instance, in the avian gastro-intestinal tract, there is a crop at the lower end of the esophagus. This functions both to store feed and for microbial action. The avian immune system lacks lymph nodes and has a distinct organ producing B-lymphocytes, namely the bursa *Fabricius*. The important of spleen has been largely dismissed until recently. However, its importance in both innate and specific immunity is increasingly recognized. There is a major difference between birds and mammals is the female reproductive system as birds produce large yolk filled eggs. The precursors of the yolk are synthesized by the liver. Another difference is that there is a single ovary and oviduct in birds.

Keywords: avian, domestication, ovary, oviduct, bursa, spleen

OPEN ACCESS

Edited by:
George E. Billman, The Ohio State University, United States

Reviewed by:
Takeshi Ohkubo, Ibaraki University, Japan
Gregoy Y. Bedecarrats, University of Guelph, Canada

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Specialty section:
This article was submitted to Avian Physiology, a section of the journal Frontiers in Physiology

Received: *12 March 2020*
Accepted: *09 October 2020*
Published: *09 November 2020*

Citation:
Scanes CG (2020) Avian Physiology: Are Birds Simply Feathered Mammals? Front. Physiol. 11:542466. doi: 10.3389/fphys.2020.542466

INTRODUCTION

The physiology of birds has attracted significant attention. A caveat is that much of the research on the physiology birds has been with domesticated birds, particularly chickens and ducks. The present communication discusses examples from the following systems where birds differ from mammals: gastrointestinal tract and specifically the crop and ceca, immune system and specifically the bursa *Fabricius* and spleen and female reproduction. Moreover, a series of questions are asked. It is noted that the physiology of birds reflects impacts of their evolutionary history, effects of domestication and the *sequentia* of flight.

Evolutionary Relationships

Birds and mammals have been long separated. The *Synapsida* (mammals and extinct ancestors) and *Reptilia* (encompassing turtles, lizards, crocodiles, dinosaurs, and birds) diverged 318 million years ago (MYA) (Benton et al., 2015). Common features of all birds, or at least their ancestors, are the following: the ability to fly, the presence of feathers and the production of large yolky eggs with thick shells. Birds are much closer to lizards, snakes (last common ancestor – 256 MYA) and particularly crocodiles (last common ancestor – 247 MYA) than they are to mammals (Benton et al., 2015). Birds evolved from bipedal dinosaurs with the first true bird thought to have existed at the end of the Jurassic period/beginning of the Cretaceous period (Brusatte et al., 2015). Based on genomics, the last common ancestors were the following:

- *Palaeognathae* (ostriches, emus, tinamous, etc.) and the *Neognathae* (all other birds) – 113 MYA during Cretaceous period.
- *Neoaves* (virtually all birds today) and *Galloanseres* (ducks, geese, chickens, pheasants and their kin) 88 MYA during Cretaceous period.
- The ancestors of the major groups of birds including land birds and water birds diverged at about the time of the Cretaceous–Paleogene (K-Pg) boundary (66 MYA) with some diverging before and some immediately after (Brusatte et al., 2015).

An identifiable fossil land bird has been described from ∼62.5 million-year-old rocks (Ksepka et al., 2017). An "explosive" radiation of groups of birds occurred shortly after the K-Pg boundary (Ksepka et al., 2017).

Domestication and Selection by Humans

There is clear evidence that domestication and later selection has influenced the genetics and phenotype of poultry. Chickens were domesticated from members of the *Gallus* genus beginning at least 8000 years ago (West and Zhou, 1988) with multiple domestication events in South Asia, Southwest China and Southeast Asia (Miao et al., 2017). The genetics of today's chickens reflect genetics coming from red jungle fowl (*Gallus gallus*) together with introgressions from the green jungle fowl (*G. varius*) (Sawai et al., 2010) and the gray jungle fowl (*G. sonneratii*) presumably after domestication. Prior to scientific selection, there were also shifts in the genetics and hence physiology of poultry. For instance, based on studies with native chickens from Africa and Europe, chickens from different regions are genetically equipped for different environmental temperatures (Fleming et al., 2017). Breeds of domesticated poultry were recognized considerably over 100 years ago; the development of these reflecting genetic drift and hardiness within specific locals together human intervention.

Caveats

White Leghorns are frequently used as a surrogate for all chickens or even all birds (e.g., Roth and Lind, 2013; Fallahsharoudi et al., 2015; Løtvedt et al., 2017) but the sources of White Leghorns vary as does their genetics. Another issue is that commercial breeding of broiler chickens, laying chickens, turkeys, and ducks is closely held within primary breeding companies with the genetics "protected." The lines are subjected to intense selection focusing on commercially important parameters such as growth rate, egg production, and efficiency. An example of the changes in genetics is the over four-fold increase in growth rates in meat-type chickens (**Table 1**). Similarly, increases in growth rate have been reported by Havenstein et al. (1994, 2003) comparing, respectively, 1991 and 2001 meat-type chickens with random bred chickens. There have continued to be improvements in growth rate since 2005. The genetics of the birds differ even for lines having the same name due to selection and use of different grandparent lines. This is very different from situation with inbred rodent lines.

Impact of Domestication and Selection by Humans

It is increasingly recognized that successful domestication was accompanied by shifts in genetics, and hence physiology. Domestication alone or with later selection was associated with shifts in the responses to stress including within the hypothalamic pituitary axis (Fallahsharoudi et al., 2015; Løtvedt et al., 2017). Differences in the stress physiology have been reported between chickens of a major egg laying breed (White Leghorns) and wild Red Jungle fowl (*Gallus gallus*) with depressed basal circulating concentrations of pregnenolone and dehydroepiandrosterone (DHEA) together with circulating concentrations of corticosterone following restraint in domesticated chickens (Fallahsharoudi et al., 2015). In addition, there is increased expression of the following stress related genes under both basal or stress conditions in the hypothalamus of domesticated chickens: *CRHR1*, *AVP*, and *GR* (Løtvedt et al., 2017). There have shifts in the eye after domestication and selection with, for example, red jungle fowl having greater optical sensitivities at low light intensities than White Leghorn chickens (Roth and Lind, 2013). Moreover, pea-comb mutation is related to SOX5 (Wright D. et al., 2009) leads to reduced comb and wattle size and, thereby, leading to reduction in susceptibility to lesions following freezing temperatures (reviewed: Wright D. et al., 2009).

Shifts in supposedly "domestication related" genes have been reported for yellow skin color (β-carotene dioxygenase 2) and thyroid-stimulating hormone receptor (*TSHR*) (a missense mutation from glycine to arginine) and wild-type allele (Rubin et al., 2010; Karlsson et al., 2015). However, these mutations appear to occurred within the past 500 years rather than at the time of domestication (Garland Flink et al., 2014).

Differences in Body Weight Between Mammals and Birds

Mammals and birds have the same organ systems but there are differences (these being discussed below). **Table 1** shows relative weights for critical organs in mammals and birds together with blood flow. The relative weights of heart, liver and kidneys differ between mammals and birds (**Table 1**) being increased for heart (increased by 3.54-fold), liver (decreased by 47.7%) and kidneys (increased by 56.1%) in birds. Spleen relative weights

TABLE 1 | Effect of genetic selection of growth in meat-type chickens (Zuidhof et al., 2014).

Average daily gaing d^{-1}	Chickens	
Between days	**Control[#]**	**2005 commercial meat-type**
1–7	4.6 ± 0.15^a	15.9 ± 0.15^b
22–28	15.3 ± 0.65^a	81.9 ± 0.65^b
36–42	20.6 ± 1.04^a	99.6 ± 1.04^b
50–56	23.0 ± 1.65^a	101.1 ± 1.65^b

[a,b]Different superscript letters indicate difference from control $p < 0.05$.
[#]Random bred – not subject to scientific selection.

are markedly lower (73.9%) in birds than mammals (**Table 2**). Blood flow is similar between mammals and that in the, albeit low number of birds examined (**Table 1**). **Question 1: What accounts for the higher relative weights of the heart, kidneys and liver? Question 1: Are they related to lower efficiency of avian systems or to specific needs of, respectively, flight, uric acid excretion and egg production?**

GASTROINTESTINAL TRACT

The gastrointestinal tract of birds shows close similarities to that of mammals with, for instance, an esophagus for the passage of ingesta from the mouth to the equivalent of the stomach and a small intestine made up of duodenum, jejunum and ileum where much enzymatic digestion and virtually all of absorption occurs and a colon (large intestine). Moreover, the liver and pancreas play similar roles supplying, respectively, bile with bile salts and pancreatic juice with digestive enzymes. There are also differences including the following, using chickens and ducks as exemplars:

1. The absence of teeth and hence chewing.
2. The presence of the crop as an outgrowth of the lower esophagus.
3. The separation of the enzymatic and muscular aspects of the stomach into the proventriculus and gizzard.
4. The presence of two large ceca.
5. The small size of the colon.
6. The presence of a common exit for urine and feces.
7. The retrograde flow of ingesta with urine from the cloaca through the colon.

Two avian features will be considered in more detail, namely the crop and ceca.

Crop

Based on studies in chickens and turkeys, the crop can act as a storage organ for feed. While there is little feed in the crop during the day, ingesta are present in the crop throughout the scotophase (e.g., Scanes et al., 1987; Buyse et al., 1993; Johannsen et al., 2005) when feeding is not occurring (references) (**Table 3**). The amount of feed in the crop progressively declines during the night (e.g., Scanes et al., 1987; Buyse et al., 1993; Johannsen et al., 2005) (**Table 3**). Thus, the situation appears to be that the chickens and turkeys gorge in the late afternoon (Scanes et al., 1987; Buyse et al., 1993) with the feed stored in the crop to be released during the nocturnal fast. Similarly, there is storage of feed in meal fed meat-type sexually immature female chickens (broiler breeder pullets) with approximately double stored when fed on alternate days (de Beer et al., 2008). A case can also be made for the proventriculus/gizzard being a site for feed storage (**Table 3**).

Both the crop developed and the stomach separated into two distinct anatomical features (the gizzard and proventriculus); occurring during avian evolution and with the arrangement has been retained in multiple taxonomic groups. **Question: What is the selective advantages if these?**

The ability of the hoatzin (*Opisthocomus hoazin*) to ferment plant materials is well established (Grajal et al., 1989) with the presence of rumen-like methanogens confirmed (Wright A.D. et al., 2009). Some consider that the hoatzin is the only avian fore-gut fermenter (e.g., Wright A.D. et al., 2009). However, there is evidence for crop fermentation with the extended nocturnal storage of ingesta (see **Table 3**). During the night when chickens and turkeys do not eat (reference), there is a gradual release of ingesta (see **Table 3**). There are also decreases in soluble carbohydrate (**Table 3**) (laying hen: Scanes et al., 1987) and increases in the concentrations of organic acids, predominantly lactic acid/lactate (**Table 3**) (turkey poult: Johannsen et al., 2005). There was not evidence for production of the major volatile fatty acids (VFAs): acetic acid/acetate, propionic acid/propionate or butyric acid/butyrate (turkey poult: Johannsen et al., 2005). What is not clear is whether and, if so, the rate to which, lactic acid/lactate and other fatty acids are absorbed from the crop? Interestingly, there is evidence that the crop plays a role in calcium absorption in laying hens with reduced egg production and serum calcium concentrations following cropectomy (Stonerock et al., 1975).

There are also marked increases in the lactic acid/lactate concentrations of the ingesta from the crop to proventriculus/

TABLE 2 | Comparison of the relative weights of and blood flow to major organs in mammals and birds.

Organ	Relative organ weight%[P]		Blood flow ml min^{-1} g^{-1}	
	Mammals	Birds	Mammals[Q]	Birds [duck[R]] (chicken[S])
Brain	0.999 ± (6) 0.321	1.469 ± (8) 0.426	0.88 ± (8) 0.11	0.84 [0.84]
Heart	0.706 ± (6) 0.052	2.497 ± (11) 0.299***	3.29 ± (7) 0.85	3.94 [2.69] (5.28)
Liver	2.831 ± (6) 0.378	1.481 ± (11) 0.212**	0.42 ± (6) 0.15	0.62 [0.58] (0.67)
Kidney	0.620 ± (6) 0.095	0.968 ± (11) 0.090*	3.83 ± (7) 0.36	4.43 [1.08] (7.78)
Spleen	0.336 ± (5) 0.038	0.0877 ± (4) 0.0115***	2.02 ± (7) 0.63	4.16 [5.56] (2.77)

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to mammals.
[P]Data is the mean of the means of mammalian or avian orders (n = the number of orders) ± SEM].
[Q]Based on mean ± (n = number of species) SEM. [‡]Data from dogs (Li et al., 1989), Mongolian gerbil (Matsumoto et al., 1982), mouse (Wang et al., 1993), rabbit (Neutze et al., 1968), pigs (neonatal Undar et al., 1999; Thein et al., 2003), rats (Alexander et al., 1972; Ishise et al., 1980; Sakanashi et al., 1987; Adán et al., 1994), rhesus monkey (infant: Behrman and Lees, 1971), and sheep (fetal: Tan et al., 1997) (neonatal: Alexander et al., 1972).
[R]Data from Jones et al. (1979) and Kaul et al. (1983).
[S]Sapirstein and Hartman (1959); Boelkins et al. (1973), and Merrill et al. (1981).

TABLE 3 | Changes in the crop attributes during the scotophase.

	Time relative to the beginning of the scotophase (night/darkness)		
	0→+1 h	**+5 h**	**+9→10 h**
Laying hen[p]			
Crop contents g	46.0 ± 5.4[c]	18.1 ± 4.1[b]	1.9 ± 1.0[c]
Soluble carbohydrate% of dry wt. of contents	17.1 ± 0.5[a]	18.3 ± 4.2[a]	8.5 ± 1.1[b]
Proventriculus/gizzard contents g	28.8 ± 2.6[c]	21.4 ± 1.3[b]	15.5 ± 1.5[a]
Young meat-type chicken[q]			
Crop contents g	28.4 ± 6.9[c]	11.6 ± 3.5[b]	0.2 ± 0.08[c]
Proventriculus/gizzard contents g	22.2 ± 3.0[b]	11.0 ± 1.9[a]	7.4 ± 1.5[a]
Turkey poult[r]			
Contents g	22.2 ± 2.4[a]	22.4 ± 3.9[a]	13.2 ± 2.3[b]
Moisture%	39.4 ± 2.3[a]	29.5 ± 1.4[b]	29.7 ± 2.1[b]
pH	5.9 ± 0.1[a]	5.0 ± 0.2[b]	5.0 ± 0.2[b]
Lactic acid μMoles g^{-1}	13.4 ± 4.5[a]	93.3 ± 17.4[b]	98.4 ± 11.4[b]
Caproic acid (C6) μMoles g^{-1}	0.17 ± 0.02[a]	0.87 ± 0.28[b]	1.33 ± 0.06[b]
Valeric acid (C5) μMoles g^{-1}	0.13 ± 0.04[a]	0.11 ± 0.03[a]	0.83 ± 0.11[b]

[a,b,c]*Different superscript letters indicate difference $p < 0.05$.*
[p]*Scanes et al. (1987).*
[q]*Buyse et al. (1993).*
[r]*Johannsen et al. (2005).*

TABLE 4 | Proportion of individual VFAs generated in the ceca of chickens.

	Young meat-type chickens[X]	**Adult female chickens**[Y]
Acetic acid/acetate (C2)	76.9	51.6 ± (10) 2.8
Propionic acid/propionate (C3)	5.9	26.7 ± (10) 1.9
Butyric acid/butyrate (C4)	17.5	9.2 ± (10) 0.8
Isobutyric acid/isobutyrate (C4)	ND	1.3 ± (10) 0.4
Isovaleric acid (C5)/2 methyl butyric acid	ND	1.8 ± (10) 0.5
Valeric acid/valerate (C5)	ND	2.0 ± (10) 0.4

[X]*Kumar et al. (2018).*
[Y]*Calculated from data in Annison et al. (1968) for laying hens.*
ND, not detected.

TABLE 5 | Generation by methane and nitrous oxide by poultry.

Species	**Methane generated**	**Nitrous oxide generated**
	mg bird^{-1} day^{-1}[A]	**mg bird^{-1} day^{-1}**[A]
Muscovy duck	26.4	
Mule	17.4	
Domesticated goose	11.4	0.45[B]
	kg head^{-1} life cycle^{-1}[BC]	kg head^{-1} life cycle^{-1}[BC]
Broiler chickens	1.59×10^{-5}	3×10^{-8}
Village chickens	8.48×10^{-5}	1.635×10^{-5}
Geese	1.5×10^{-3}	4.90×10^{-5}

[A]*Chen et al. (2014).*
[B]*Chen et al. (2003).*
[C]*Wang and Huang (2005).*

gizzard and along the small intestine (domestic goose: Clemens et al., 1975). This may reflect microbial fermentation or anaerobic metabolism by gut tissues. Given that there is extended storage of feed in the proventriculus/gizzard during the night (**Table 3**), it is not clear whether there is also fermentation in the proventriculus/gizzard. **Question: How much lactic acid and other potential nutrients absorbed from the crop?**

Ceca

The ceca are major sites of fermentation with production of VFAs with the concentration of VFAs of 107.3 ± (3) 4.1 μMoles g^{-1} in the cecal contents (laying hen: Annison et al., 1968). **Table 4** summarizes the contribution of individual VFAs in the colon contents of chickens. Acetic acid/acetate is the dominant VFA. The VFA concentrations of cecal content concentrations increase following feeding rising from ∼20 μmoles ml^{-1} 2 h after feeding to ∼70 μmoles ml^{-1} 8 h after feeding (domestic goose: Clemens et al., 1975). The ceca are also the major site of methane production by, at least geese; with methane production being reduced by 91% in cecectomized geese (Chen et al., 2003) (**Table 5**). In contrast, nitrous oxide emission from geese is not influenced by cecectomy (Chen et al., 2003) (**Table 5**). Addition of alfalfa can reduce methane production by over 70% in Muscovy ducks, mule ducks and white Roman geese (Chen et al., 2014). Interestingly, there was no overall effect of addition of antibiotics to feed on VFA production by chickens (Kumar et al., 2018).

What is not clear is whether and, if so, the rate to which, VFAs are absorbed from the ceca? The concentrations of VFA in the ingesta have been reported to decrease in the colon and cloaca (Clemens et al., 1975). This arguably indicates absorption. The overall importance of the ceca is not supported by the lack of effect of cecectomy on growth rates in geese (Chen et al., 2003).

Question 1: What are the rates of absorption of VFAs and other nutrients from the ceca? Question 2: Is this physiologically relevant and, if not, why are the ceca so large?

IMMUNE SYSTEM

There are similarities between the avian and mammalian immune system with the presence of both innate and both T and B cell mediated specific immune responses and the presence of the thymus. However, there are marked differences between the organization of the immune system in the two classes including the following in birds:

1. The presence of the bursa Fabricius – the primary immune organ producing B cells in birds.
2. The lack of lymph nodes in birds.

Question 1: Are the structures that have yet to be described that are essentially "lymph nodes"? Question 2: What have birds to replace the functions of the lymph nodes?

Until recently, the avian spleen was largely ignored. Indeed, John (1994) concluded that the avian spleen was "little-studied" by avian physiologists. This is no longer the case with substantial interest by immunologists. Examples of advances in our understanding of the spleen are discussed below.

Bursa Fabricius

The avian bursa Fabricius played an important role in the development of understanding of immune functioning. Antibody formation is greatly reduced in chickens bursectomized at 2 weeks old (Glick et al., 1956; Ewert and Eidson, 1977). Birds that were bursectomized at 60 h of embryonic development have immunoglobulin Ig producing B cells but do not exhibit an ability to generate a specific antibody response (Mansikka et al., 1990).

Spleen

Anatomy

The avian spleen is smaller than the mammalian organ (see **Table 1**) (John, 1994). In birds, the spleen is surrounded by a connective tissue capsule (Kannan et al., 2015). The avian spleen can be considered composed of two tissues: red pulp (with blood containing sinusoids with cords of lymphocytes, macrophages, granulocytes, plasma cells, and mast cells) and white pulp (lymphoid tissue composed of young lymphoblasts, lymphocytes, follicular dendritic cells, and reticular cells) (chicken: Oláh and Glick, 1982; Kannan et al., 2015; reviewed: Powers, 2000). There are unmyelinated nerve fibers present in the ellipsoids (Oláh and Glick, 1982). Central artery is surrounded by ellipsoids (Oláh and Glick, 1982). The venous drainage from the spleen passes to the hepatic portal vein (Powers, 2000). There is evidence for a blood-spleen barrier in birds (domestic duck: Sun et al., 2019).

Roles of the Avian Spleen

The roles of the avian spleen are considered as the following:

1. As a lymphoid organ
2. Phagocytosis of senescent or damaged erythrocytes (Powers, 2000).

However, the avian spleen does not appear to be a temporary store of erythrocytes (Powers, 2000). **Question 1: If the spleen does not act as a temporary storage site for erythrocytes, is there an alternate system?**

Stressors and the Spleen

Spleen weights in birds are depressed by stress (see **Table 6**). This is irrespective of whether the stress is mimicked by the administration of corticosterone (Mehaisen et al., 2017) or represents transportation stress (Zhang et al., 2019) or protein deficiency (Adrizal et al., 2019). It is not surprising given the ability of corticosterone to depress the weight of the spleen (Mehaisen et al., 2017; also see **Table 4**) that the *MC2-R gene* is expressed in the chicken spleen (Takeuchi and Takahashi, 1998). Moreover, heat stress markedly increased the incidence of lesions in the spleen of broiler chickens (Aguanta et al., 2018). Similarly, corticosterone decreases the weights of the primary immune organs, thymus and bursa Fabricius, in birds (e.g., young chickens: Mehaisen et al., 2017). Spleen weights are also decreased following exposure to toxicants (Aflatoxin B_1: Grozeva et al., 2017; Ochratoxin A: Khan et al., 2019; T-2 toxin: Hayes and Wobeser, 1983) (see **Table 6**). In contrast, spleen weights were increased after viral challenges or administration of *E. coli* lipopolysaccharide (see **Table 6**) (Boa-Amponsem et al., 1999; Zhang et al., 2017; Bai et al., 2019; Yang et al., 2019).

Effects of Splenectomy

There is evidence that the avian spleen has both positive and negative effects on immunity. Splenectomy decreased primary immune response (the titer of antisera) after intravenous challenge with sheep red blood cells (Hippeläinen and Naukkarinen, 1990). Paradoxically, splenectomy improved

TABLE 6 | Effect of challenges on spleen weights or relative spleen weights (data is expressed as the percentage of the control ± SEM).

Treatment	Species	Control	Treated	Calculated from reference
Corticosterone[n]	Young chickens	100 ± 5^b	68 ± 12^a	Mehaisen et al. (2017)
Transportation stress[#]	Young chickens	100 ± 3^b	88 ± 3^a	Zhang et al. (2019)
Low protein feed	Young chickens	100 ± 6^b	76 ± 6^a	Adrizal et al. (2019)
Aflatoxin B_1	Turkey poults	100 ± 15^b	71 ± 7^a	Grozeva et al. (2017)
Ochratoxin A[#p]	Young chickens	100 ± 9^b	52 ± 4^a	Khan et al. (2019)
T-2 toxin[#q]	Mallard ducks	100 ± 2	83 ± 3	Hayes and Wobeser (1983)
LPS[r]	Young chickens	100 ± 2^a	119 ± 2^b	Yang et al. (2019)
Low energy feed and LPS	Yong ducks	100 ± 6^a	112 ± 6^b	Bai et al. (2019)
Marble spleen disease virus[#]	Young chickens	100 ± 5^a	136 ± 6^b	Boa-Amponsem et al. (1999)
Chicken infectious anemia virus[s]	Young (SPF) chickens	100 ± 13^a	161 ± 31^b	Zhang et al. (2017)
Marek's disease virus[s]		100 ± 13^a	132 ± 14^b	Zhang et al. (2017)

[a,b] *Different superscript letters indicate difference from control $p < 0.05$.*
[#] *Relative spleen weights.*
[n] *Daily i.m. injections of 0.5 mg kg^{-1} for 7 days.*
[p] *Subcutaneous administration.*
[q] *Treatment for 2 weeks.*
[r] *E. coli lipopolysaccharides injected i.p. on alternate days × 4.*
[s] *After 9 days.*

TABLE 7 | Relative weights of the ovary and oviduct in ducks and chickens together with blood flow [data is shown as mean ± (number of studies) SEM].

Tissue	Ovary	Oviduct	References
Relative weight%			
Sexually immature chickens	0.046 ± (4) 0.15[a]	0.046 ± (2) 0.24[a]	Maurice et al. (1982); Sun et al. (2006); Martínez et al. (2015); Dunn et al. (2017)
Sexually mature ducks	2.45	2.47	White et al. (1978)
Sexually mature chickens	2.42 ± (13) 0.22[b]	4.165 ± (7) 0.95[b]	Brody et al. (1984); Kwakkel et al. (1995); Joseph et al. (2000); Sun et al. (2006); Chen et al. (2007); Emiola et al. (2011); Pishnamazi et al. (2014); Saki et al. (2014); Sun et al. (2015); Hassan et al. (2016); Youssef et al. (2016); Nassar et al. (2017)
Blood flow ml min^{-1}			
Sexually mature chickens	5.05 ± (3) 1.12	24.0 ± (6) 4.37	Boelkins et al. (1973); Moynihan and Edwards (1975); Niezgoda et al. (1979); Scanes et al. (1982); Hrabia et al. (2005); Rząsa et al. (2008)

[a,b]*Different superscript letters indicates difference $p < 0.05$.*

the response of turkey poults challenged with hemorrhagic enteritis virus (HEV); decreasing the incidence of hemorrhagic diarrhea and mortalities (Ossa et al., 1983).

The Avian Spleen and Immune Functioning

The avian spleen plays important roles in both innate and specific immune responses. *STING* (stimulator of interferon genes) is expressed in the spleen (chicken: Ran et al., 2018). Infection of specific pathogen-free chickens with Newcastle Disease Virus was followed by increased expression of STING together with that of interferon (INF)-α, INF-β, and Interferon Regulatory Factor 7 (IRF-7) in the spleen (Ran et al., 2018). Rous sarcoma virus (RSV) infection of susceptible chickens is followed by increased expression of pro-inflammatory cytokines such as in spleen including interleukin (IL) 8 and IL 10. Moreover, there is marked expression of the Th1 cytokines IFN-γ and TNF-α in the spleen (Khare et al., 2019). In pigeons, infection with Newcastle disease viruses increases expression of *RIG-I*, *IL-6, IL-1*β, *CCL5*, and *IL-8* genes in the spleen (Xiang et al., 2019). LPS challenge increased antioxidant capacity with elevated malondialdehyde (MDA) concentrations in chicken spleen (Yang et al., 2019). There is high expression of toll-like receptor (TLR) 5 in the spleen and peripheral blood mononuclear cells (pigeon: Xiong et al., 2018). In the presence of flagellin (from Gram-negative bacteria), there is increased expression of TLR5, interleukin (IL)-6, IL-8, CCL5, IFN-γ and NF-κB by pigeon splenocytes (pigeon: Xiong et al., 2018). Administration of a synthetic ligand for TLR21 increased expression of IFNα but decreased that of IL-6 in the chicken spleen (Sajewicz-Krukowska et al., 2017). Chickens infected with Marek's disease virus (Gallid herpesvirus 2) have more γδ T cells in their spleens (Laursen et al., 2018). Spread of Marek's disease virus (MDV) to the spleen and thymus is delayed in Ig heavy chain J gene segment knockout (JH-KO) chickens lacking mature and peripheral B cells (Bertzbach et al., 2018). *Escherichia coli* infection is accompanied by decreased splenic expression of antibacterial nucleotide-binding oligomerization domain-like receptor (NLR) pyrin domain containing 3 (NLRP3) (Li et al., 2018). **Question 1: Is there redundancy such that the spleen is not necessary in birds?**

FEMALE REPRODUCTION

The avian egg is large with a yolk filled ovum, surrounded by egg white, membranes and a shell composed of calcium carbonate. Yolk is composed of the following:

- Water – ∼50%
- Solids – ∼50% composed of the following:
  - Low-density fraction (∼65% of yolk solids)
  - Granules (∼25% of yolk solids) composed of the following:
    - Lipovitellin (a lipoprotein formed by the cleavage of vitellogenin in the oocyte)
    - Phosvitin (a phosphoprotein formed by the cleavage of vitellogenin in the oocyte)
  - Water soluble or proteins in the aqueous fraction – the livetins (∼10% of yolk solids) composed of the following:
    - α Livetins – Synonymous with blood serum albumen
    - β Livetins – Synonymous with blood serum α_2-globulin presumably containing transport proteins
    - γ Livetins – Synonymous with blood serum γ -globulin specifically immunoglobulin Y (IgY) (equivalent to IgG in mammals).

The yolk precursors, vitellogenin (VTG) and very low density lipoproteins (VLDL), are synthesized in the liver under the estrogen stimulus (Deeley et al., 1975; reviewed Loh et al., 2011). They pass to the ovary via the circulatory system and their transport into the oocyte mediated by the receptor VLDL/VTG receptor (VLDL/VTGR) (chickens: Steyrer et al., 1990; Stifani et al., 1990; Barber et al., 1991; Bujo et al., 1994). Restricted ovulator chickens have a mutation in the VLDLR//VTGR and exhibit markedly reduced follicular development with elevated circulating concentrations of phospholipids, triglyceride and cholesterol (Elkin et al., 2003, 2012). However, there was still yolk deposition in the presence of the mutant VLDLR (Elkin et al., 2003, 2012). This suggests the existence of an alternate mechanism for deposition of the yolk precursors.

Question 1: Are there alternate mechanisms for transport of yolk precursors into the oocyte?

There is little information on the transport of livetins into the developing oocyte. The γ livetins are almost exclusively (97.7%) IgY (Hamal et al., 2006; Agrawal et al., 2016). **Question 2: What is the mechanism for the transport of IgY into the oocyte? Question 3: What is the mechanism for selection of IgY versus IgM and IgA? Question 4: To what extent does this reflect limits on molecular size for proteins passing into the interstitial space?** The concentrations of albumen and γ-globulin in the interstitial space of chickens were greater than those of α-globulin and β-globulin when expressed as a percentage of vascular concentrations (calculated from data in Peltonen and Sankari, 2011). The granulosa cell layer and the tight junctions between cells may act in an analogous manner differentially permitting some, but not other, proteins to transit. There is much higher expression of the tight junction protein, occludin, by granulosa cells from smaller white follicles than large yellow follicles and being absent in preovulatory follicles (Schuster et al., 2004). The transport of cations into the yolk has received little attention. It is reasonable to assume that transport of calcium into the yolk occurs along with vitellogenin with calcium bound to the phosphate moieties. Sodium concentrations in the aqueous fraction are 44.6 mEquiv. L^{-1} (Gilbert, 1971; Grau et al., 1979). This is markedly higher than reported intracellular concentrations of sodium (erythrocyte: 13.6 mEquiv. L^{-1}) and lower than the plasma concentration (Miseta et al., 1993; reviewed: Scanes, 2015). **Question 5: What are the mechanisms for sodium and other cations transport across the oocyte membrane?** At least some of the sodium is likely to enter along with VTG during endocytosis but then why isn't sodium pump out?

In most birds, there is a single ovary (the left) and the left Müllerian duct develops into an organ called the oviduct. However, in a few species such as kiwis, there are paired ovaries and oviducts (Kinsky, 1971). The oviduct is made up of the following: infundibulum, magnum, isthmus, shell gland or uterus and vagina. Neither the oviduct nor uterus are equivalent to their name-sakes in mammals although both are derived from the Müllerian duct. The ovary is larger than that of mammalian ovaries with the ovary having a relative weight in sexually mature chickens of 2.42 ± (number of studies = 13) SEM 0.22% of body weight and sexually mature ducks 2.45% of body weight (see **Table 7**). This is due to the yolk accumulating in the oocyte within the follicle with the yolk precursors synthesized in the liver (see above).

The relative weights of avian ovaries and oviducts are shown in **Table 7**. There are marked increases in both between sexually immature and mature female chickens with increases of 52-fold for the ovary and 91-fold for the oviduct (**Table 7**). There is high blood flow to both the ovary and oviduct (**Table 7**). There are also differences in blood flow to regions of the oviduct with higher blood flow to the magnum 9.56 ± (6) 2.49 ml min^{-1} and shell gland 10.1 ± (6) 2.42 ml min^{-1} than the infundibulum 1.01 ± (5) 0.10 ml min^{-1}, isthmus 2.47 ± (6) 0.40 ml min^{-1} and vagina 0.96 ± (2) 0.20 ml min^{-1} (Boelkins et al., 1973; Moynihan and Edwards, 1975; Niezgoda et al., 1979; Scanes et al., 1982; Hrabia et al., 2005; Rząsa et al., 2008).

There are several studies on the effects of neurotransmitters on blood flow to the ovary and oviduct in birds. Histamine increased blood flow to stroma, small white follicles, large yellow follicles and post-ovulatory follicles together with the infundibulum and shell gland. In addition, histamine increased cardiac output (Hrabia et al., 2005). Serotonin induces a transient decrease (after 1 min) in blood flow to small white follicles and F4 and F5 large yellow follicles and to the shell gland with blood flow restored to at least pretreatment after 5 min (Rząsa et al., 2008). Similarly, prostaglandin $F_{2\alpha}$ decreases blood flow to the large yellow follicles (Scanes et al., 1982). What is missing, are studies of the role of the nervous systems. **Question 1: What are the roles of the nervous system in the control of ovarian and oviductal functioning? Question 2: The control of blood flow to ovary and oviduct warrants further attention. Question 4: What is not clear is the extent blood flow reflect the metabolic requirements of a tissue or is blood flow a driving force dictating or restricting the metabolism of tissues?**

There are substantial loads placed on the female bird in synthesizing the proteins of egg white proteins, the membranes and the shell. In female birds, calcium is stored in a short-term basis in medullary bone in long bones. Calcium is mobilized from this storage in an attempt to balance the outflow of calcium in the uterus (shell gland) forming the egg. **Question: How is calcium mobilized from medullary bone?** In addition to parathyroid hormone, and 1,25 dihydroxy vitamin D_3 (Castillo et al., 1979), there appears to be an involvement of other mechanisms. There is increased expression of receptor activator of nuclear factor-kB (RANK) and fibroblast growth factor (FGF23) in medullary bone of hens peaking at the end of egg calcification and the end of calcium mobilization from the medullary bone (Gloux et al., 2020). There was also increased expression of solute carrier family 20 member 1 (SLC20A1) and member 2 (SLC20A2) (Gloux et al., 2020). Moreover, circulating concentrations of phosphate are elevated in laying hens passively immunized against FGF23 (Ren et al., 2019).

The mechanism for calcium transfer to the shell has received considerable attention. It is mediated at least in part by calbindin D 28K (formerly known as vitamin D-dependent calcium-binding protein) (Bar et al., 1996). Uterine expression of calbindin D 28K is increased by estrogens if 1,25 dihydroxy vitamin D3 is present (Nys et al., 1992). Androgens potentiate the ability of estrogens to increase expression of calbindin D 28K in the uterus during sexual maturation (Nys et al., 1989). Moreover, there are shifts in uterine gene expression of as the ovum passed down the oviduct with, as might be expected, increased expression of calbindin D 28K and transient receptor potential vanilloid channel type 6 (TRPV6) when the egg is being calcified (Nys et al., 1989; Yang et al., 2013). Concentrations of CaBP-D28k protein in the uterine mucosa are depressed in the presence of either interleukin-1β and interleukin-6 *in vitro* (Nii et al., 2018). **Question 1: What are other mechanisms, if any, for calcium transport?**

CONCLUSION

In avian physiology, there are a series of assumptions employed that may or may not be valid. Examples of these assumptions include the following:

- Domesticated birds provide little or no information about wild birds as the former have been subjected to intensive anthropomorphic selection.
- The contrary view is that the physiology of one species of bird, domesticated or wild, are readily transferable to another.

The debate between these views is accentuated by some rigidity of those with either an ornithological or poultry orientation. This situation is confounded by the lack of common meetings, departments and education. The differences in education include the following:

- The lack of courses (particularly at the graduate level) experienced by poultry physiologists on wild birds and on the ungirding principle of biology, namely evolution.
- There is a corollary with appreciation for poultry lacking in those studying wild birds.

Another erroneous assumption is that birds are merely "feathered" mammals with a few specific differences related, for instance, to flight and production of large yolky eggs. Instead, the physiology of birds reflects their long evolutionary history.

Finally, to adapt the quotation from both George Santayana and Winston Churchill, "*Those who fail to learn from or even read the literature including the older literature are doomed to repeat the studies and not advance science.*"

AUTHOR CONTRIBUTIONS

This is solely the work of CS.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

SPECIALTY GRAND CHALLENGE
published: 28 February 2020
doi: 10.3389/fphys.2020.00186

Frontiers in Invertebrate Physiology—An Update to the Grand Challenge

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Keywords: genomic basis of physiology, plasticity of invertebrate physiology, adaptation to anthropogenic changes, nematode, *Drosophila*, ecophysiology, comparative physiology

OPEN ACCESS

Edited by:
George E. Billman, The Ohio State University, United States

Reviewed by:
Robert Huber, Bowling Green State University, United States

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Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *14 January 2020*
Accepted: *18 February 2020*
Published: *28 February 2020*

Citation:
Anton S, Gadenne C and Marion-Poll F (2020) Frontiers in Invertebrate Physiology—An Update to the Grand Challenge. Front. Physiol. 11:186. doi: 10.3389/fphys.2020.00186

"Studying invertebrate physiology is an exciting domain, because it provides on one hand insight into general principles of animal physiology, utilizing models with different degrees of complexity and on the other hand it allows studying evolutionary adaptations to a multitude of different lifestyles. Environmental constraints and basic construction principles have led to an amazing variation of physiological solutions to breathe, to ingest and digest food, to reproduce and to communicate and all this on the basis of a wide range of anatomical construction principles. Therefore, a comparative approach to invertebrate physiology is extremely rich and can only be encouraged. The goal of Frontiers in Invertebrate Physiology is to cover a wide range of approaches from the molecular to the cellular, organismic, and even the population level. Studies on model and non-model organisms and on all aspects of physiology will be published, to provide a forum for exchange of recent advances in the field.

Invertebrates represent 95% of all living animal species. They have colonized all habitats on earth, including Polar regions, deserts, and seas. Their external skeleton (at least for some of the most prominent invertebrate groups) and their segmented central nervous system make them unique models to study developmental physiology (e.g., molting processes) and the gradual architectural evolution of their central nervous system, and the resulting neural and sensory physiology. Moreover, many invertebrate species are organized in very sophisticated societies, thus offering exciting challenges to study the physiology of intra- and inter-specific interactions and their adaptation to environmental constraints (Woodard et al., 2011)."

GENOMIC SEARCH FOR INVERTEBRATE PHYSIOLOGICAL FUNCTIONS

Ten years of Frontiers in Invertebrate Physiology with an increasing number of submissions every year, illustrate the great progress in general in invertebrate physiology, and more specifically in integrating molecular advances with physiological function. After the extremely ambitious i5k initiative launched in 2011, intending to sequence the genomes of 5,000 insect species within 5 years (Robinson et al., 2011), 155 insect genomes have now been annotated, around 400 genomes have been assembled and more than 1,200 projects have been registered in the NCBI database (Li et al., 2019).

In addition to widely used correlative approaches, linking variations in gene expression with phenotypic variations in animal behavior and physiology, large progress has been made in the development of gene knockout methods for both model and non-model organisms, such as RNAi techniques and the more recent CRISPR Cas9 system (Tijsterman et al., 2002; Sun et al., 2017). Whereas mutants with well-defined deficiencies have been used for physiological studies in model

organisms like *Drosophila melanogaster* and *Caenorhabditis elegans* for more than 40 years (Corsi et al., 2015; Kaufman, 2017), these latter approaches have recently helped to identify general physiological mechanisms down to the cellular and molecular level across species (He et al., 2019), but also uncovered variability in physiological mechanisms between different organisms. A large spectrum of Research Topics in Frontiers in Invertebrate Physiology illustrates this type of progress in multiple invertebrate taxa. Linking genomic and physiological results with invertebrate behavior requires now high throughput behavioral experiments with challenging analysis methods, involving for example machine-learning algorithms, creating the new discipline of ethomics (Geissmann et al., 2017).

INVERTEBRATE PHYSIOLOGY AND HUMAN ACTIVITIES

Another field, which has advanced importantly in the last decade is the understanding of physiological mechanisms in invertebrates with importance in human activity, such as agriculture, aquaculture, and the transmission of diseases, with the ultimate goal to control pest species or improve rearing conditions for organisms used for human and animal nutrition (Frontiers in Invertebrate Physiology Research Topics on cephalopods, insect-pathogen-plant interactions, tick-and tick-borne pathogens, insect immune systems, digestive enzymes, adaptation to environmental stresses,…). Especially the control of pest species, such as herbivores and disease vectors, urgently requires the development of alternative methods, due to important changes in legislation concerning chemical control measures all over the world (for example the restriction of the use of neonicotinoid insecticides by the European Food Safety Authority in 2018). "Studies of host-pathogen interactions, immunity, and the physiology of resistance will not only be important for food production, but also for improving public health. Although invertebrate species have been used for medical purposes for more than 4,000 years and as models for research and teaching since the end of the eighteenth century, the development of invertebrate models for e.g., neurodegenerative diseases" (i.e., fruitflies) and drug addiction is recent and in plain expansion (Sarkar et al., 2016; von Staaden and Huber, 2019). Major physiological mechanisms arose very early in animal evolution and have been highly conserved in spite of the amazing diversification of external morphology. Therefore, high throughput approaches required by modern interdisciplinary research, feasible in model invertebrates such as *D. melanogaster* and *C. elegans*, will provide a solid basis to solve important questions in vertebrate, including human and clinical physiology. "These recent developments will allow to reduce the use of mammals for medical research (e.g., drug development), important issue from an ethical and economic point of view." Environmental risks and risks for human and animal health, as well as the development of resistance of many pest species lead to important challenges in the coming decade to discover new physiological targets for pest control. Among emerging ideas for alternative pest management, reverse chemical ecology proposes new specific targets in form of olfactory proteins for pest control or conservation biology (Zhu et al., 2017; Choo et al., 2018). Another emerging target for pest control is to manipulate their digestive enzymes (Zibae, 2012).

INVERTEBRATE PHYSIOLOGY AND ANIMAL AND HUMAN NUTRITION

An interesting twist of our interest in pest insects is to consider them as a potential source of proteins and nutritive substances. Since the FAO report about entomophagy and its potential to contribute to feed animals and humans (van Huis et al., 2013), considerable efforts have been made to develop the production of a few insect species like *Tenebrio molitor* and *Hermetia illucens* at an industrial scale in Europe and in the rest of the world. While the first species is a pest studied in the laboratory since a long time, data about other species like *Hermetia illucens* are scant. There is thus a great demand of data concerning the nutrition and reproduction physiology of such species, together with data about how their food affects their nutritional quality, as well as how to keep them healthy and resistant against their own pathogens.

INVERTEBRATE ADAPTATION TO ANTHROPOGENIC ENVIRONMENTAL CHANGES

Anthropogenic environmental changes, such as climate change and pollution also open a whole new field of research in invertebrate physiology in a context of ecological and environmental questions. Ecophysiological approaches with recent advances include system biology approaches (using modeling) (Damos et al., 2018) and adaptations to stress conditions (Tang et al., 2017). Climate change and pollution have also recently been shown to change intra- and interspecific communication channels (Boullis et al., 2016; Fuentes et al., 2016; Jürgens and Bischoff, 2017). However, the field of ecophysiology is only at its beginning and major research efforts in this field are needed in the future.

NEURAL PLASTICITY OF INVERTEBRATES

"Another issue, which has been increasing enormously during the last years, is the investigation of plasticity in the nervous control of physiological functions (Yamada et al., 2010). Adaptations to external and internal modifications in sensory and motor systems controlling different physiological functions become more and more evident. Detection of intra- and inter-specific stimuli is modified by e.g., experience and reproductive state (Iyengar et al., 2010)." The last 10 years have shown important advances in the investigation of mechanisms of plasticity in invertebrates (Pyza, 2013; Vafopoulou, 2014). "However, we are far from understanding the cellular mechanisms and signaling pathways involved in these forms of physiological plasticity. It will be important in the future to investigate the role of hormones, neuromodulators, and their mechanisms of action ideally in parallel in different invertebrate species both during development and in adult organisms. New

biochemical techniques allow us to detect and measure minute traces of neuropeptides in single cells, whose roles are still largely unknown (Yew et al., 2009)." Another important point is the rapidly developing field of epigenetics, showing an important role of DNA methylation in adaptation processes (Gavery and Roberts, 2013).

All parts of the manuscript in quotation marks are taken from Anton et al. (2011).

AUTHOR CONTRIBUTIONS

SA, CG, and FM-P contributed equally to the writing of the manuscript.

ACKNOWLEDGMENTS

We thank the reviewer for helpful comments.

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