Integrative and Comparative Biology

Integrative and Comparative Biology, pp. 1–16
doi:10.1093/icb/icaa056

Society for Integrative and Comparative Biology

SYMPOSIUM

Trade-offs, Pleiotropy, and Shared Molecular Pathways: A Unified View of Constraints on Adaptation

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From the symposium "Building Bridges from Genome to Phenome: Molecules, Methods and Models" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 2020, Austin, Texas.

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Synopsis The concept of trade-offs permeates our thinking about adaptive evolution because they are exhibited at every level of biological organization, from molecular and cellular processes to organismal and ecological functions. Trade-offs inevitably arise because different traits do not occur in isolation, but instead are imbedded within complex, integrated systems that make up whole organisms. The genetic and mechanistic underpinning of trade-offs can be found in the pleiotropic nodes that occur in the biological pathways shared between traits. Yet, often trade-offs are only understood as statistical correlations, limiting the ability to evaluate the interplay between how selection and constraint interact during adaptive evolution. Here, we first review the classic paradigms in which physiologists and evolutionary biologists have studied trade-offs and highlight the ways in which network and molecular pathway approaches unify these paradigms. We discuss how these approaches allow researchers to evaluate why trade-offs arise and how selection can act to overcome trait correlations and evolutionary constraints. We argue that understanding how the conserved molecular pathways are shared between different traits and functions provides a conceptual framework for evolutionary biologists, physiologists, and molecular biologists to meaningfully work together toward the goal of understanding why correlations and trade-offs occur between traits. We briefly highlight the melanocortin system and the hormonal control of osmoregulation as two case studies where an understanding of shared molecular pathways reveals why trade-offs occur between seemingly unrelated traits. While we recognize that applying such approaches poses challenges and limitations particularly in the context of natural populations, we advocate for the view that focusing on the biological pathways responsible for trade-offs provides a unified conceptual context accessible to a broad range of integrative biologists.

Introduction

The concept of trade-offs plays a central role in biology (Stearns 1989; Kitano 2004; Roff and Fairbairn 2007; Guillaume and Otto 2012; Garland 2014; Bourg et al. 2019; Chen and Zhang 2020). Trade-offs are exhibited at every level of biological organization, from molecular and cellular processes (Flatt and Kawecki 2007; Campos et al. 2016; Sheftel et al. 2018) to organismal and ecological functions (Ghalambor et al. 2004; Simmons and Emlen 2006; Olsen et al. 2019). Biological trade-offs can generally be defined as the condition when a beneficial change in one trait or function results in a detrimental change to another trait or function (Stearns 1989). The presence of such trade-offs inevitably arise because different traits, functions, phenotypes, and almost all biological processes do not occur in isolation, but instead are imbedded within highly integrated and hierarchical systems and networks that make up whole organisms (Wagner and Altenberg 1996; Wagner and Zhang 2011; Hill and Zhang 2012; Murren 2012; Bourg et al. 2019). In this context, biological trade-offs are no different than those found in any complex system, in that multiple interacting parts must work together to carry out particular functions. Yet, such complexity and

June 1, 2020
Published by Oxford University Press on behalf of the Society for Integrative and Comparative Biology 2020.

integration also leads to a fundamental dilemma often referred to as the "cost of complexity"; when many interacting parts are needed to successfully carry out a function, changing any one part will inevitably negatively impact other traits, altering function and potentially reducing overall performance or fitness (e.g., Wagner and Altenberg 1996; Orr 2000a; Welch and Waxman 2003; Wainwright et al. 2005; Wagner et al. 2008). For example, consider the complexity of all the interacting elements of the mTOR (mamalian target of rapamycin) signaling pathway, that receives and integrates signaling molecules from a wide range of environmental stimuli (e.g., nutritional status, hypoxia, insulin, stress, and growth factors) and in turn interacts with numerous proteins to control many of the most fundamental cellular processes (reviewed in Kim and Guan 2019; Liu and Sabatini 2020). Such complexity represents a major challenge in attempts to understand the diverse molecular, developmental, and physiological interactions that transform genetic variation into phenotypic variation (Burnett et al. 2020a), or what is referred to as the genotype–phenotype map (e.g., Alberch 1991; Wagner and Altenberg 1996; Wagner 2007; Rockman 2008; Wagner and Zhang 2011).

The relationship between genetic variation and phenotypic variation is an inherently non-linear one, as a diversity of causal factors must jointly interact with each other to carry out all basic functions. The genotype–phenotype map is a nontrivial and fundamental challenge because it requires understanding how variation in individual genes and interactions across the genome (e.g., epistasis and pleiotropy) are propagated via molecular interactions that originate in individual cells and feed into various molecular pathways and networks to determine complex phenotypes (Soyer 2012). Yet, despite the recognition that the genotype–phenotype map requires embracing the complexity of highly integrated organismal systems, empirical approaches have largely struggled to capture the degree of interconnectedness between different levels of biological organization (i.e., from genes to proteins to functional phenotypes) and between different mechanisms (e.g., mutation, transcription, RNA editing, protein function, etc.). Instead, the study of mechanisms that transform genomic variation into phenotypic variation is dominated by reductionist approaches that reduce the overwhelming complexity into more manageable components. For example, advances in cellular and molecular biology now allow an understanding of the independent and collective contributions of gene products (e.g., transcriptomes and proteomes), the regulatory control of these products (e.g., hormones, cytokines, and other signaling molecules), how these products interact (e.g., protein–protein interactions) and ultimately the pathways these products participate in and the functions they determine (Lodish et al. 2008). These approaches have generated large data sets and made significant progress toward understanding the set of genes, proteins, and other molecules that make up certain pathways, networks, and phenotypes (e.g., signal transduction, gene regulation, and metabolic pathways). However, far less attention has been given to how these mechanisms and pathways interact and result in trade-offs at the level of whole organisms (Arnold 1992; Solovieff et al. 2013; Sommer and Mayer 2015). Here, we are interested in the mechanistic basis of trade-offs and advocate for incorporating the study of molecular pathways into evolutionary and physiological approaches to better understand the causes and constraints imposed by trade-offs. We first present an overview of the traditional and emerging conceptual frameworks used by evolutionary biologists and physiologists to study and understand the mechanistic basis for trade-offs and how these trade-offs ultimately constrain adaptive evolutionary change. We argue that these complimentary approaches find common ground when they focus on the degree to which molecular pathways and networks are shared between different functions and on the origins of these shared pathways. We then highlight some established and emerging models for studying how shared pathways result in trade-offs between different functions, and the consequences for constraining adaptive evolution.

Traditional frameworks for studying pleiotropy and trade-offs

Evolutionary biologists have long been interested in the presence, causes, and consequences of trade-offs and recognize that many traits are not inherited independently, but rather as genetically correlated or linked traits that can trade-off against one another. In "The Genetical Theory of Natural Selection" Ronald Fisher was perhaps the first to explicitly relate trade-offs and organismal complexity with his geometric model of adaptive evolution (Fisher 1930). Fisher argued that organisms were like microscopes, in that they operated best when all possible adjustments (think the turning of the different knobs of a microscope) were done in harmony, such that a mutation (or adjustment of a knob) would more likely be beneficial if it had a small effect size (i.e.,

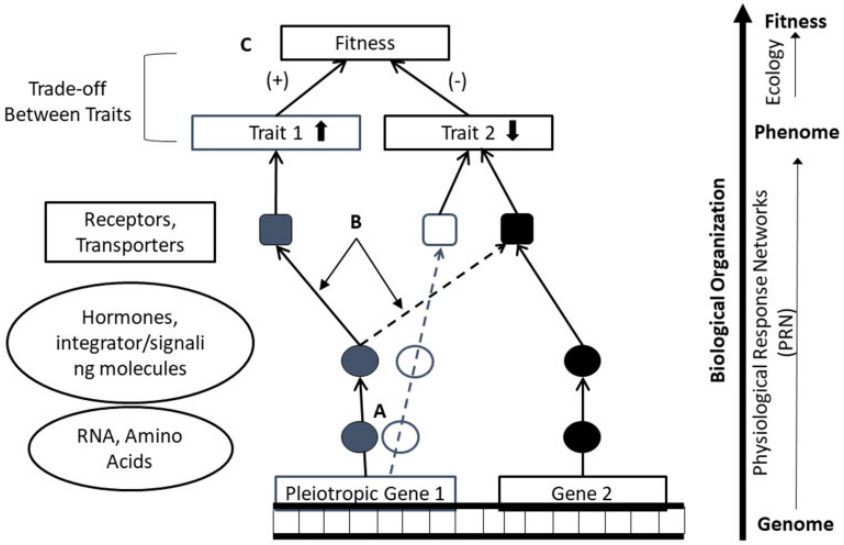

Fig. 1 A simplified conceptual model of trade-offs in a genome to phenome context. The figure demonstrates two non-mutually exclusive ways in which a trade-off can arise via pleiotropy. (**A**) denotes how "genetic pleiotropy" can arise if pleiotropic gene 1 has two molecular functions (compare solid line versus dashed line) that are part of two separate pathways leading to Taits 1 and 2. In this case, the filled and open circles are representative of gene products (mRNA, signaling molecules, hormones) that interact with downstream targets represented by filled and open squares (e.g., receptors). In this case, the consequence of the pleiotropic gene is antagonistic because it increases the value of Trait 1 which increases fitness, while decreasing the value of Trait 2 which lowers fitness. (**B**) denotes "hormonal pleiotropy," where a single signaling molecule like a hormone (light black circle) binds with two different receptors (filled light black and dark black squares), which in turn also increase Trait 1, while decreasing Trait 2. Because this hormone is ultimately a product of gene 1, both situations could be defined as genetic pleiotropy. This distinction may not be readily recognized in studies that only focus on one level of biological system, that is, genetic systems or the endocrine system. However, if the focus is on the pathways instead, the different paths by which Traits 1 and 2 are influenced by gene 1 (as shown by the dashed and solid lines) become evident. (**C**) denotes the fitness consequence of antagonistic pleiotropy, where increasing Trait 1 increases fitness (e.g., producing more offspring) and decreasing Trait 2 reduces fitness (e.g., reduced survival). The contrasting fitness consequences of this type of pleiotropy are context dependent and likely to change in response to spatial and temporal environmental variation. Lastly, the location along the genome to phenome to fitness map at which these different pathway components (e.g., genes, gene products, hormones, receptors, traits, and fitness) can be measured is depicted on the right side of the figure. As one progresses along with this map, one also moves up in biological organization (i.e., cells to tissue to whole organism to organism interactions) and this is depicted alongside the map.

a small adjustment as opposed to large adjustment of the knob) because it would be less likely to have a large impact on multiple traits and compromise function. Implicit in Fisher's model was the concept of pleiotropy: the ability for a single gene to affect more than one trait (see Fig. 1 for a simple example of genetic pleiotropy where a single gene affects two traits; Hodgkin 1998; Paaby and Rockman 2013). Pleiotropy describes the pattern by which different traits and functions are genetically connected or correlated, and how pleiotropy manifests itself in a system determines the pattern of trade-offs and constraints. For example, under the idea of "universal pleiotropy" any mutation at any locus has the potential to affect every trait either directly or indirectly. Under the assumption of universal pleiotropy, the cost of complexity is high as trade-offs between traits would be very common. Conversely, if pleiotropy is rare, then the cost to complexity is alleviated because traits are free to change without impacting other traits or functions (Stearns 2010; Paaby and Rockman 2013).

In the context of trade-offs, the primary focus of evolutionary biologists has been on antagonistic pleiotropy, or when a single gene affects two traits in opposing directions, such that increasing one trait value results in a decrease in the value of the other trait (with increased trait values being associated with fitness; Paaby and Rockman 2013). For example, Fig. 1 illustrates a scenario where a pleiotropic gene codes for two distinct molecular functions that result in an increase in Trait 1 that increases fitness and a decrease in Trait 2 which decreases fitness: antagonistic pleiotropy. The presence of antagonistic pleiotropy has far reaching implications because it provides an explanation for why natural selection is unable to simultaneously optimize multiple traits and how improvement in one function can cause reduced fitness through correlated changes in other functions. Testing for the presence of antagonistic

pleiotropy has historically relied on a correlative and statistical approach based on breeding and artificial selection experiments to estimate the degree to which different traits are genetically correlated with each other (Stearns 2010). Such approaches have found unexpected genetic correlations among traits. For example, selective breeding for high voluntary wheel-running behavior in mice has led to a suite of correlated evolutionary changes in almost every component of the phenotype from morphology (e.g., Castro and Garland 2018), physiology (e.g., Hiramatsu and Garland 2018), to the neuroendocrine system (Garland et al. 2016). Furthermore, recent studies have combined artificial selection experiments with genome-wide association studies (GWAS) to uncover candidate pleotropic genes that could underlie observed trait correlations and trade-offs (e.g., Dong et al. 2019). Collectively, such results highlight not only the magnitude of interconnectedness and integration at the whole organism level, but also the potential constraints imposed on traits under selection to independently evolve (Garland et al. 2016). The recognition of such genetically based trade-offs have been extremely influential in the design of selective breeding experiments for agricultural products (Falconer and Mackay 1996; Chen and Lübberstedt 2010), the evolutionary theories for senescence (e.g., Williams 1957), life history evolution (e.g., Roff 1992; Stearns 1992), adaptation (e.g., Bennett and Lenski 2007), and the maintenance of genetic variation (Falconer and Mackay 1996).

Physiologists have also had a long history of interest in trade-offs at multiple levels of biological organization. All physiological and biochemical processes are ultimately grounded in the constraints imposed by physical and chemical laws, which in term place limits on the range of available adaptive solutions to environmental challenges (e.g., Garland and Losos 1994; Somero et al. 2017). However, physiologists and evolutionary biologists have increasingly found common ground by investigating the mechanisms underlying how finite resources are allocated to the competing demands of growth, developmental, reproduction, and maintenance (e.g., Sibly and Calow 1986; Zera and Harshman 2001; Ricklefs and Wikelski 2002; Flatt and Heyland 2011). Such allocation trade-offs commonly underlie life history and developmental trade-offs. For example, Zera et al. (1998) experimentally demonstrated the role of juvenile hormone (JH) in controlling the allocation of nutrients between ovaries and flight muscle; an allocation trade-off that underlies two distinct life history strategies associated with early reproduction versus flight capability. Similarly, JH also plays a role in the allocation of resources to different body parts during development, such as between the size of beetle horns and eyes (Nijhout and Emlen 1998). The multiple roles of JH exemplify a general role played by hormones in generating trait correlations and trade-offs (Zera et al. 2007; McGlothlin and Ketterson 2008; Ketterson et al. 2009; Hau and Wingfield 2011; Cox et al. 2016; Dantzer and Swanson 2017). How hormones act pleiotropically on diverse suites of traits encompassing morphology, physiology, and behavior, and how changes in hormone receptors can alter tissue sensitivity to break up these pleiotropic effects are major themes in the emerging field of evolutionary endocrinology (Zera et al. 2007; McGlothlin and Ketterson 2008; Ketterson et al. 2009; Hau and Wingfield 2011; Cox et al. 2016; Dantzer and Swanson 2017). An example of hormonal pleiotropy is also depicted in Fig. 1 where one hormone interacts with receptors that underly with the two different traits. Now, one can argue that this is still just an example of genetic pleiotropy as the hormone originates from a single gene. However, by understanding the pathway responsible for the trade-off, one gains a more precise understanding of the roles played by different gene products and where in the pathway the trade-off originates. The development of evolutionary endocrinology as a field of study reveals that physiologists and evolutionary biologists are converging on a shared and more integrative understanding of the mechanisms that underlie trade-offs.

Trade-offs and pleiotropy in the context of network theory

Recent developments in next-generation sequencing, mass spectrometry, and other technologies have generated vast amounts of high dimensional data at different levels of biological organization (genomes, transcriptomes, proteomes, etc.), which in turn has led to new perspectives and interest in the role of pleiotropy and trade-offs. In particular, there has been a shift toward embracing perspectives based on complex systems and network theory to study patterns within large data sets, as in evolutionary systems biology (Soyer 2012; Soyer and O'Malley 2013; Melo et al. 2016) or in the context of physiological response networks (PRNs; Cohen et al. 2012; Martin and Cohen 2015). These network approaches attempt to mathematically model the complex pattern of interactions underling the relationship between genomes and phenomes (Soyer 2012; Soyer and O'Malley 2013; Melo et al. 2016). Systems biology tools are used to dissect these properties so

researchers can understand network dynamics and make informed predictions about how complex systems will respond to mutations or other perturbations (Soyer 2012; Soyer and O'Malley 2013; Ciaccio et al. 2014).

The topological structure of networks shares common features such as individual nodes (often specific molecules or genes) that are connected to each other by edges that represent physical interactions or genetic processes, and emergent features such as modularity (e.g., functionally similar nodes that strongly interact with each other) and robustness (i.e., the ability of the network to maintain function in response to genetic or environmental perturbations; Soyer and O'Malley 2013; Ciaccio et al. 2014; Melo et al. 2016). These basic features of networks can be seen in Fig. 1: signaling molecules, receptors, and genes represent nodes that are connected to one another via physical interaction (signaling molecule to receptor) or genetic processes (genes to receptors/molecules). In the context of trade-offs and evolutionary constraints, modularity is a key concept because it describes the degree to which traits are constrained or free to evolve within complex networks. Specifically, a system is modular when it "can be divided into multiple sets of strongly interacting parts that are relatively autonomous with respect to each other" (Melo et al. 2016). In other words, traits within a module (e.g., levels of gene expression, parts of a morphological trait) are highly correlated and thus constrained from independently changing due to pleiotropic interactions and shared underlying pathways, whereas separate modules are more independent of each other and free to change without compromising particular functions. Properties of networks/modules, the degree of connectivity between modules, and the way in which crosstalk occurs between modules, influence how organisms respond to environmental change and influence how modules respond to selection (Martin et al. 2011; Melo et al. 2016). Indeed, the presence of modularity is thought to be one solution to the cost of complexity (see above) because it allows for complex systems to be compartmentalized into subnetworks that minimize negative pleiotropic effects (Wagner and Altenberg 1996; Wagner 2007; Melo et al. 2016; Dantzer and Swanson 2017).

But how common is pleiotropy throughout the genome and what is the evidence that pleiotropy is more common within than between modules? How much does pleiotropy result in trade-offs that constrain adaptive evolution and does modularity actually lessen the constraint? New genomic tools and network analyses are providing empirical measurements of the patterns of pleiotropy across genomes. Wagner and Zhang (2011) summarized genomic studies on yeast, nematodes, mice, and humans and found that pleiotropic effects were largely modular—meaning that most pleiotropy was found within the same functional gene networks. The finding that specific biological functions have integrated genetic underpinnings was not new (e.g., Chesler et al. 2005; Wagner et al. 2007) but finding that pleiotropy was largely confined to integrated networks suggests that pleiotropically based trade-offs should be limited to similar biological functions (Wagner and Zhang 2011). A surprising conclusion was that pleiotropy seemed to be low across the genome (the median mutation in these studies only affected 2–8% of the traits examined; Wagner and Zhang 2011). Wagner and Zhang (2011) also found that the per-trait effect size of a mutation increased with the number of traits affected by the mutation. In their model, this meant that "moderately" complex organisms had faster adaptation rates than the simplest of organisms, counter to the "cost of complexity" paradigm. Wagner and Zhang (2011) suggest that trade-offs and evolutionary constraints due to pleiotropy may not be as prominent as once thought. However, Hill and Zhang (2012) warned against any broad conclusions because the statistical framework used by Wagner and Zhang (2011) may have underestimated pleiotropy. Furthermore, the trait changes analyzed by Wagner and Zhang (2011) were not necessarily linked to a fitness change. Traits closely linked to fitness may have a different prevalence than other forms of pleiotropy because of the complex effects of trait–trait interactions on fitness (Paaby and Rockman 2013). The directionality of this difference will be challenging to test because the fitness consequences of trait changes are inherently difficult to measure and highly dependent on environmental context (Eguchi et al. 2019). Nevertheless, the degree and form of pleiotropy across the genome remains an open but critical question not only in the context of evolvability, but also in our ability to associate genetic variants to specific phenotypes (Boyle et al. 2017).

Physiologists have also started to incorporate network thinking by viewing physiological systems as complex networks. Viewing physiological responses as a network instead of a series of individual responses leads to explicitly considering how different physiological systems interact with each other and enables the ability to anticipate potential trade-offs. Cohen et al. (2012) defined PRNs as "the network of molecules and their regulatory relationships

that maintain and adjust homeostasis and facilitate performance at the whole-organism level." Like other types of networks, PRNs have nodes and edges, where the nodes are typically the circulating concentrations of signaling molecules (e.g., hormones, proteins, mRNA, etc.) and their associated receptors, whereas the edges define the co-regulatory patterns of the nodes—such as the magnitude and direction of change in one node after another node has been altered (Martin and Cohen 2015). What is appealing about PRNs is that they shift the scale of biological organization to the whole organism and identify the mechanistic interactions between whole physiological systems (Cohen et al. 2012; Martin and Cohen 2015). In this context, PRNs view physiological systems as being dynamic (i.e., having a PRN state), such that PRN states will shift in order to achieve homeostasis or carry out a particular function (Cohen et al. 2012; Martin and Cohen 2015). Despite the appeal of PRNs as providing a more holistic view of how physiological states respond to environmental variation through plasticity and evolutionary change, the specific architecture of most PRNs remains largely unknown (Martin and Cohen 2015). Nevertheless, a PRN approach is particularly useful in stimulating more critical thinking about the exact mechanisms that generate trade-offs, and how those trade-offs might be resolved. For example, both Flatt and Heyland (2011) and Hughes and Leips (2018) note that not all resource allocation trade-offs are necessarily determined by energy budgets but could alternatively be explained by the way signaling pathways are shared between different life history traits. Thus, understanding PRN architecture can help compare alternative hypotheses for why trade-offs arise between different physiological systems.

By viewing physiologically based trade-offs in the context of modularity and PRNs, a link can be made between how physiologically based trade-offs constrain or facilitate adaptive evolution. The relation to evolutionary thinking is two-sided: the rate of evolution could be slowed and the potential direction of evolution within trait space could be limited by trait correlations created by networks, or alternatively, tight trait correlations that are products of past selection could facilitate rapid adaptive change (Ketterson et al. 2009). For example, Aubin-Horth et al. found that boldness and aggression were correlated in sticklebacks because the traits share an underlying biological network (Aubin-Horth et al. 2012). If an environmental change jointly selects for increased boldness and aggression, then perhaps adaptive evolution would be facilitated due to the shared network. But if an environmental change requires breaking these two traits apart, adaptive evolution could be constrained. This type of network thinking can help identify spots in a physiological network where alterations would be most effective in ameliorating trade-offs. Indeed, Di Poi et al. (2016) studied four PRNs in freshwater and marine stickleback populations and found that adaptation to freshwater was facilitated by changing patterns of expression in specific receptors rather than global changes to the entire PRN, providing empirical evidence for the idea that modularity in biological pathways can ameliorate trade-offs due to pleiotropy. Similarly, Sommer and Mayer (2015) reviewed developmental mechanisms in different nematodes and found a conserved "developmental switch" controlling a hormone receptor. The switch is used in many ecological functions and was the evolutionary target for the development of novel regulatory loops that altered intraspecific competition (Sommer and Mayer 2015). This switch is an example of an "integrator" (Cohen et al. 2012), a part of a network that connects modules and is disproportionally important in shaping trade-offs (Martin et al. 2011) and thus is often implicated as being key to creating or ameliorating trade-offs.

Studying pleiotropy and trade-offs in the context of molecular pathways

The conceptual frameworks used by evolutionary biologists and physiologists to study pleiotropy and trade-offs share one unifying component: the biological pathways that underlie the traits of interest determine the degree to which the traits are correlated. The mechanisms by which pleiotropy and trade-offs are manifested within organisms can be understood by focusing on the molecular pathways that are involved in the transmission of signals, the regulation of gene expression, and/or metabolism and how they all interact as part of a larger network (Soyer 2012). Yet, how pleiotropy manifests itself at the molecular level is complex (Fig. 1), as single genes may have different molecular functions depending on context or have a single molecular function that impacts multiple biological functions (Dudley et al. 2005; He and Zhang 2006; Paaby and Rockman 2013; Stoney et al. 2015). Indeed, when one considers how the molecular function of genes change as one examines processes at the levels of single cells, tissues, or organs, and how these changes impact the physiological state of whole organisms, the definition of pleiotropy, traits, and trade-offs changes at different levels of biological organization (Paaby and

Rockman 2013; Stoney et al. 2015). These molecular mechanisms are also dynamic and often activated and regulated by hormones in response to changing internal and external environmental conditions (e.g., Aranda and Pascual 2001; Cheung and Kraus 2010). Nevertheless, by focusing on the molecular pathways influencing the traits of interest, genetic, and hormonal pleiotropy converge upon studying the same set of mechanisms for the causes and consequences of trade-offs.

Evolutionary biologists and physiologists embracing molecular pathways have started to gain new insights into the causal mechanisms underlying correlations, trade-offs, and constraints in a diversity of phenotypic traits closely tied to fitness (e.g., Flatt et al. 2005; Ayroles et al. 2009; Chen and Lübberstedt 2010; Hau and Wingfield 2011; Schwartz and Bronikowski 2011; Schwartz and Bronikowski 2013; Aubin-Horth 2016; Saltz et al. 2017; Durmaz et al. 2019). Such integrative approaches have been dependent on advances made in cell and molecular biology and are increasingly available to a broader range of researchers because genes, gene functions, and biological pathways are largely conserved across multicellular organisms. While much work remains in understanding the complexities of gene function and molecular pathways, integrative biologists working with nonmodel organisms can use widely available databases to annotate gene functions (e.g., gene ontology "GO," see Ashburner et al. 2000) and tools for pathway enrichment analysis (e.g., KEGG (Kyoto Encyclopedia of Genes and Genomes), MetaCyc, Reactome, see Bindea et al. 2009; Kelder et al. 2012; Altman et al. 2013; Mykles et al. 2016; and for recommendations for future studies see Burnett et al. 2020b). The challenge in most cases will be to connect the action of molecules and the associated biological pathways with ecologically relevant fitness related traits (Loewe 2012). But when these challenges are overcome, such approaches can lead to novel discoveries. For example, Ihle et al. (2015) investigated the mechanistic control of a complex behavioral syndrome in honeybees. Previous work had shown that two mutually suppressive hormones, JH and vitellogenin (Vg), were largely responsible for mediating the pollen hoarding syndrome (PHS), a syndrome in which foraging, ovary size, and life history traits are all linked. Ihle et al. (2015) selected for strains that differed in their strength of the PHS, then functionally reduced Vg via RNA interference in a backcross of different strains and conducted a QTL study on various phenotypes of the PHS. This methodology leads to the discovery of the genetic basis for endocrine controlled traits. Using a different approach, Ayroles et al. (2009) used transcriptome sequencing in 40 different inbred fruit fly lines to understand the genetic underpinnings of, among other things, six correlated phenotypic traits. They found statistically significant trade-offs between many of the traits (e.g., competition and starvation resistance and longevity and competition). They used modularity clustering techniques (Stone and Ayroles 2009) to break the traits into modules of transcripts and then looked for genes that overlapped between modules (at a rate higher than expected by chance). The researchers discovered "substantial modular pleiotropy" and used GO terms to better understand the functionality of the pleiotropic genes. For example, they found that genes affecting mitochondrial ribosomes to be pleiotropic for chill coma recovery and starvation resistance; a pattern that was otherwise not obvious. In other cases, knowledge of existing molecular pathways can be applied to better understand particular systems. Schwartz and Bronikowski (2013) provide a model approach for studying how molecular stress pathways shape life history evolution and the specific nodes within this pathway that are under selection. Similarly, Regan et al. (2020) describe how the insulin like signaling pathway and related pathways such as mTOR, integrate a range of environmental inputs and provide a robust framework for understanding the relationship between dietary restriction and aging. Collectively, these approaches provide evidence for the power of focusing on the molecular pathways that connect gene functions to phenotypes, as opposed to focusing only on statistical association between specific candidate genes and phenotypes (e.g., GWAS studies; see also Boyle et al. 2017). Inspired by this work, below we briefly highlight the melanocortin system and the control of osmoregulation, as different ways in which molecular pathways can be used to study trade-offs and make *a priori* predictions about evolutionary constraints in a genome to phenome context.

The melanocortin system: Trade-offs between coloration and behavior

Variation in color and its correlation with other traits have been important in ecological and evolutionary studies as color-related traits are known to respond to natural selection in the wild (Hoekstra et al. 2004; Hoekstra et al. 2005), artificial selection in the lab (Rajpurohit et al. 2016), and have even been implicated as a potential mechanism for speciation (McKinnon and Pierotti 2010). The

melanocortin system is of particular interest because it is highly conserved across vertebrates, affects many traits in addition to color (e.g., aggressiveness, sexual behavior, immune function, the stress response, energy homeostasis, and social behavior; see Ducrest et al. 2008), and does so across many different tissue types (Cone 2005; Ducrest et al. 2008; Roulin et al. 2011). Additionally, the genetic basis of the melanocortin system is relatively simple as evident by small mutations that dramatically alter coloration (Hoekstra et al. 2006) and can be manipulated in the lab (Matsuoka and Monteiro 2018). Overall, it is an ideal system to study trade-offs and the pathways that underly them.

The melanocortin system refers to a set of hormonal, neuropeptidergic, and paracrine signaling pathways that are defined by various components including the five G protein-coupled melanocortin receptors (e.g., MC1-5R); peptide agonists derived from the proopiomelanocortin preprohormone precursor (e.g., melanocyte stimulating hormone (MSH) isoforms and adrenocorticotropic hormone [ACTH]) which is coded for by the *POMC* gene; and the endogenous antagonists, agouti signaling protein (ASIP) and agouti-related protein (reviewed in Cone 2006). POMC is largely expressed in the pituitary gland, so melanocortins are dispersed into the bloodstream, brain, and peripheral tissues (like skin) (Dijkstra et al. 2017), which means that different receptors can be expressed simultaneously across different tissues. Hence, in the absence of downstream regulation or mutations, the expression of the receptors and their corresponding effects on phenotypes should be correlated. For example, in fish alpha-MSH leads to the expression of MC1R in the skin, which leads to darker coloration, but it can also lead to the expression of MC3R & MC4R in the brain which can lead to increases in aggression by modulating the dopamine system (Dijkstra et al. 2017).

Because of the highly interconnected nature of the traits within the melanocortin system, and because the melanocortin system is highly conserved across vertebrates, *a priori* predictions can be made across a wide range of taxa for which traits will be correlated with color differences. Ducrest et al. (2008) did this in a meta-analysis and found darker colored individuals were often more aggressive and had a stronger stress response relative to their lighter colored conspecifics (Ducrest et al. 2008). Importantly, they were also were able to make insights as to under what conditions trait correlations between coloration and melanocortin-based phenotypes should or should not exist. Specifically, Ducrest et al. (2008) suggest that trait correlations should only exist when agonists causing the expression of MC1R in the skin (the cause of darker coloration) are coordinated with the expression of the other MCR subtypes across different tissues. Hence, differences in tissue-specific expression of inverse agonists or mutations in MC1R can lead to individuals that only differ in coloration and not any of the potentially correlated traits. For example, in the beach mouse system where color variation between populations allow them to adaptively match the substrate they live on, a significant amount of the differences in color can be attributed to a single mutation in the *MC1R* gene (Hoekstra et al. 2006) and thus the color polymorphism would not be predicted to correlate with other traits. However, when more of the melanocortin module is involved in trait expression and genetic backgrounds differ, the picture becomes more complicated. For example, Dijkstra et al. (2017) studied the role of the melanocortin system in regulating color and aggression in two color morphs of the cichlid, *Astatotilapia burtoni*. They found the same phenotypic correlation between darker color and aggression in both morphs, but they also discovered that the melanocortin system is differentially activated in the two morphs after pharmacological manipulation of alpha-MSH and ASIP (Dijkstra et al. 2017). This suggests that breaking apart the aggression/color trait correlation would need to take different routes in the two morphs which means the corresponding evolutionary constraint could be morph specific. Similarly, morph specific regulation of the melanocortin system in the Tawny Owl, *Strix aluco*, has also been found (Roulin et al. 2011; Emaresi et al. 2013; Emaresi et al. 2014). In this system, darker male owls have higher survival than their lighter conspecifics and, as predicted by life history theory, produce lower numbers of higher quality offspring than lighter males (Emaresi et al. 2014). Interestingly, investment in offspring is relatively inflexible to stress in dark males but is flexible to stress in light males, revealing a phenotypic correlation between color, life-history, and the stress response. The authors postulated that this could be due to differences in POMC regulation because previous work showed that lighter female tawny owls had altered regulation of POMC to stress manipulation, whereas their darker counterparts did not (Roulin et al. 2011). However, they have also found sex-specific regulation of melanocortin which could further complicate the matter (Emaresi et al. 2013). These examples of the melanocortin system reveal how pathways are shared between traits like color, the immune system, and life history leading to morph and sex-specific differences. Collectively, a

deeper understanding of the melanocortin system and its main components has helped increase our understanding of why seemingly unrelated traits are correlated with each other. This provides greater power to predict *a priori* when trait correlations should occur and when they might be broken which is imperative when assessing the degree to which trade-offs/trait correlations act as evolutionary constraints.

A trade-off between salinity tolerance and aggression

Osmoregulation, the process in which organisms maintain internal ion balance, is vital to aquatic organisms that experience different salinities and has been particularly well studied in fish (Marshall and Grosell 2006; Evans et al. 2013). The physiological mechanisms underlying osmoregulation in euryhaline fish have long been recognized to be under hormonal control (reviewed in McCormick 2001; McCormick and Bradshaw 2006; Sakamoto and McCormick 2006; Mancera and Mccormick 2007). Yet, the same endocrine control mechanisms are also known to have pleiotropic effects on numerous other traits (e.g., Mommsen et al. 1999). For example, arginine vasotocin (AVT), growth hormone (GH), and cortisol (McCormick 2001; Warne et al. 2002; McCormick and Bradshaw 2006; Sakamoto and McCormick 2006; Mancera and Mccormick 2007) are also known to influence behavioral aggression (Jönsson and Björnsson 2002; Santangelo and Bass 2006) and metabolism (Sangiao-Alvarellos *et al.* 2004), leading to potential trade-offs between osmoregulation and other traits (Gilmour et al. 2005; Alcaraz et al. 2008).

How might such trade-offs arise via biological pathways and how might they manifest themselves in an ecological setting? We recently began investigating if a trade-off between salinity tolerance and competitive ability in *Poecilia reticulata*, the Trinidadian Guppy, could provide an explanation for why this euryhaline fish is restricted to freshwater on the island of Trinidad. The guppy is native to the Caribbean, Central America, and South America (Magurran 2005), and has been experimentally shown to tolerate, reproduce in, and evolve improved tolerance to saltwater (Gibson and Hirst 1955; Shikano and Fujio 1998; Shikano et al. 2001). Furthermore, it can be found in brackish waters in introduced parts of its range (Courtenay et al. 1974). Yet, the guppy is only found in freshwater in the streams of Trinidad despite its documented physiological tolerance to brackish and saltwater. Sampling of the abiotic and biotic factors on Trinidad shows that the guppy's range limit is best correlated with a change in salinity and the presence of another closely related Poecilid, *Poecilia picta* or the Swamp Guppy (Torres-Dowdall et al. 2013). Hence, the movement of guppies into brackish water in Trinidad would mean increased interactions with a potential competitor in addition to dealing with increased osmoregulatory demands (see Fig. 2 for an illustration of how the change from freshwater to brackish water results in a cascade of physiological responses).

The guppy's natural history and distribution suggest that the species may experience a trade-off between aggression (a proxy for competitive ability) and osmoregulation (Torres-Dowdall et al. 2013). But why should osmoregulation and aggression be correlated traits that trade-off against one another? We hypothesize that the answer to this question for guppies and other euryhaline teleosts is due to the overlap between osmoregulatory pathways and aggression pathways. To our knowledge, no study to date has thoroughly examined how the two biological pathways controlling osmoregulation and aggression overlap to create this trade-off, although these pathways have been well studied separately. In Fig. 2, we depict a simplified model of how this trade-off may manifest via the overlap of pathways involving the pleiotropic effects of several hormones. Briefly, an increase in environmental salinity is known to cause an increase of AVT in the brain (McCormick 2001; Evans et al. 2005; Evans and Somero 2008; Filby et al. 2010; Martos-Sitcha et al. 2019). AVT synthesis and secretion result in the release of ACTH which controls the secretion of cortisol, which along with GH and other hormones, increase ion transport via activation of the NKCC ion transporter and other transporters in the gills (AVT affects the ion transporters directly too) (Mancera and Mccormick 2007; Morando et al. 2009; Lema et al. 2019; Martos-Sitcha et al. 2019). However, cortisol also acts to decrease aggression (DiBattista et al. 2005) which creates a potential for a trade-off between salinity tolerance and aggression. Interestingly, GH has been shown to increase aggression in fish (Jönsson and Björnsson 2002; Trainor and Hofmann 2006), thus it is able to increase both aggression and salinity tolerance and could perhaps ameliorate the magnitude of this trade-off. However, there is a limit to how much GH can alter the trade-off. The initial increase in AVT upon entering salinity also leads to activation of the serotonin and dopamine pathways. This is beneficial in terms of ion transport as the neurotransmitters 5-

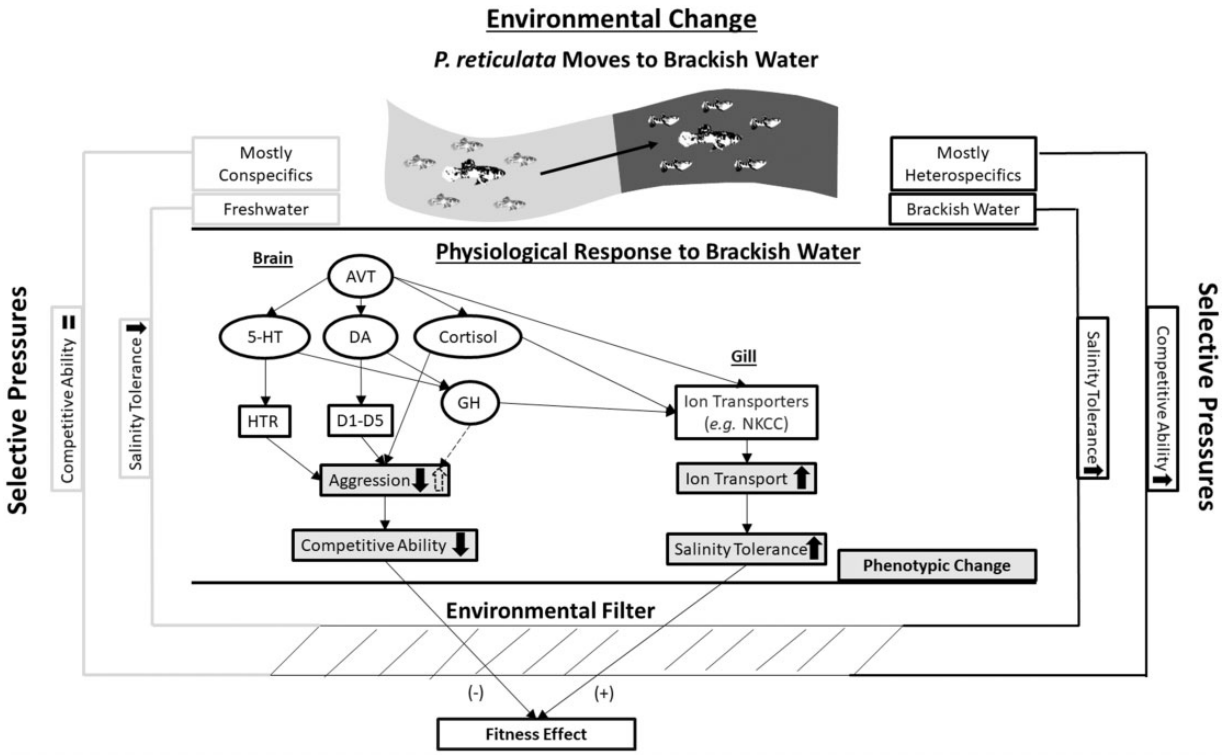

Fig. 2 A simplified pictorial summary of a hypothesized trade-off between aggression and salinity tolerance in the ecological context of the guppy, *P. reticulata*, moving from freshwater to brackish water. If individual guppies were to move from freshwater to brackish water in Trinidad, then they would also encounter swamp guppies, *P. picta*, in addition to the change in salinity. The osmoregulatory response to the increase in salinity is an increase ion transport in the gills. This response is driven by AVT, cortisol, and GH, which act as integrators or signaling molecules (denoted as circles), and their interactions with receptors/transporters (denoted as squares). These hormones act pleiotropically through shared pathways and lead to a correlation between decreased aggression and increased salinity tolerance. Note that GH is shown to increase aggression, denoted by its connection to the large upward-pointing dashed arrow within the "aggression" filled-square (see text for a more thorough discussion of the pathway). The fitness consequences of these pleiotropic effects are determined by the environmental filter, which suggests guppies cannot simultaneously tolerate increased salinity and cope with the challenge of a closely related competitor.

HT (part of the serotonin pathway) and DA (part of dopamine pathway) regulate the release of GH. But, this has a simultaneous negative effect in terms of aggression, as 5-HT activates 5-HT receptors (HTR) and DA activates DA receptors (D1–D5) that reduce aggression (Sangiao-Alvarellos et al. 2004; DiBattista et al. 2005; Gilmour et al. 2005; Filby et al. 2010; Jeffrey et al. 2014). Hence, AVT through the intermediates of cortisol, GH, 5-HT, and DA appears to act as an antagonistic pleiotropic node. There are more candidate pleiotropic nodes that could lead to the same trade-off, but preliminary evidence from experiments that measure changes in gene expression in the brain and gills most strongly implicates the pathway described above (Mauro and Ghalambor, manuscript in preparation). The same preliminary experiments also reveal that exposure to salinity challenge results in reduced aggression at the phenotypic level (Mauro and Ghalambor, manuscript in preparation). By combining transcriptomics, with experimental manipulations of salinity, and the competitive environment, we are attempting to link differential gene expression and shared pathways to the phenotypes that cause a trade-off between aggression and salinity tolerance. Although many of the details on the pathways underlying this trade-off require further investigation, we believe this approach can yield insights as to the degree this trade-off ultimately represents an evolutionary constraint.

Discussion and conclusions

There is an increasing recognition that genetically based trade-offs, like those that arise because of pleiotropy, ultimately manifest themselves through complex biological pathways to shape whole organism physiology and fitness (Bourg et al. 2019). As a result of this and by examining how genomes map to phenomes, evolutionary biologists, and physiologists are converging on a shared understanding of the molecular mechanisms underlying trade-offs and the

constraints on the range of possible phenotypes. This is in contrast to the historical conceptual divide between how evolutionary biologists and physiologists have typically studied trade-offs. Evolutionary biologists have often treated the molecular mechanisms underlying trade-offs like a "black box," the details of which were not critical to the testing and development of theory (see discussion in Flatt and Heyland 2011 with Stearns). On the other hand, physiologists, cellular, and molecular biologists have often operated outside of evolutionary theory; motivated to understand "how things work" as opposed to why they work one way instead of another (Ghalambor et al. 2015). This has led to reductionist approaches which reduce the complexity of interactions occurring within the whole organism or with the external environment (Cohen et al. 2012; Martin et al. 2011) and prevented thinking about biological pathways in an evolutionary context (Soyer 2012). But now, the fields are converging and there are an increasing number of studies demonstrating how understanding the molecular mechanisms underlying trait correlations, trade-offs, and the related networks (i.e., the shared molecular pathways) provides critical insights into the targets of selection and the degree to which selection can or cannot break trade-offs (Chen and Lübberstedt 2010; Schwartz and Bronikowski 2011; Aubin-Horth et al. 2012; Schwartz and Bronikowski 2013; Aubin-Horth 2016; Saltz et al. 2017). Our point here is that a focus on understanding the pleiotropic consequences of shared molecular pathways can facilitate integrative biologists to use a similar evolutionary framework across levels of biological organization (e.g., Mykles et al. 2010; Soyer 2012). This shared perspective will be rooted in network and systems thinking and will emphasize how genomes, molecules, and biological pathways interact to generate the molecular architecture of trait correlations and PRNs and how these networks bias evolutionary responses to natural selection (Wagner et al. 2007; Stone and Ayroles 2009; Martin et al. 2011; Ciaccio et al. 2014; Sommer and Mayer 2015). Our hope is that such approaches will move past correlative evidence for why trade-offs occur and provide a more satisfying explanation for how and why traits are correlated (e.g., Ihle et al. 2015). Most importantly, such an approach allows for *a priori* hypotheses to be generated about the ecological and evolutionary conditions that should result in selection acting at specific points/nodes within the network to overcome trade-offs. For example, one general hypothesis is that evolution to overcome trade-offs should manifest itself by altering downstream components of networks (e.g., cis elements or local rQTLs (QTLS that act on relationships between traits) in terms of genetics and receptors in terms of PRNS; Pavlicev and Wagner 2012; Pavličv and Cheverud 2015; Di Poi et al. 2016). One compelling example of this is Pavlicev and Wagner's (2012) Selection, Pleiotropy, Compensation (SPC) Model. This model suggests that directional selection will select for beneficial mutations even if these mutations have negative pleiotropic effects on fitness in other traits, because compensatory evolution in rQTLs will correct for the introduced trade-off. Under this model, increased network complexity could potentially be beneficial as more "nodes" in a network would mean more opportunity and more specificity for compensatory changes. Models like the SPC are intriguing and highlight how network architecture determines whether mutations lead to trade-offs or not.

Lastly, while we advocate for the study of molecular pathways as a way to facilitate integrative thinking about the genome to phenome map, we end on a cautionary note on the limitations and challenges that must be overcome. First, despite biological pathways being highly conserved across taxa, the multinodal networks these pathways participate in are highly plastic and offer numerous paths by which the same outcome can be reached (Tononi et al. 1999; Motegi and Seydoux 2013; Kafri et al. 2016). For example, while we have a very good understanding of the molecular mechanisms and pathways that lead from genes to shaping muscle phenotypes, as well as the selection pressures acting on these mechanisms, Hoppeler (2016) comments that modifications to the structure and function of muscles "can be achieved by an almost unlimited combination of inputs and downstream signaling events." Thus, which pathways are used to shape muscle phenotypes of different species and across diverse environments is difficult to predict, but will certainly differ from those found in model organisms (Hoppeler 2016). In this regard, muscle phenotypes are not likely to be different than any other phenotype in that a common feature of all complex networks is redundancy in parts of the system and robustness to maintain function in response to genetic or environmental perturbations (e.g., Masel and Siegal 2009; Matias Rodrigues and Wagner 2009). Thus, proper comparative studies are likely to be very insightful in terms of identifying the various ways in which selection and phylogenetic history shape network architecture. Second, the pleiotropic nature of the genome to phenome map means that the behavior of complex networks will be highly context dependent and sensitive to the internal and external environment of

the organism (e.g., Zhu et al. 2004; Papin et al. 2005; Abraham 2008; Oberhardt et al. 2009; Zhernakova et al. 2017). Similarly, the fitness consequences of trait correlations due to shared pathways will be dependent on the ecological context the organism experiences (e.g., Aubin-Horth et al. 2012; Di Poi et al. 2016). For example, the fitness consequences of the pleiotropic melanocortin system depend on the fluctuating selection pressures over time and space for coloration, aggression, and life history (see above). Similarly, a trade-off between osmoregulation and aggression due to the pleiotropic effects of hormones is predicted to only have negative fitness consequences when fish are challenged by salinity and competitive interactions (see above and Fig. 2). The context dependency of these fitness trade-offs should lead to strong selection for compensatory changes within the networks (e.g., Pavlicev and Wagner 2012), however, in fluctuating environments the cost of these compensatory changes may outweigh the benefits. We are still in the infancy of documenting how much variation exists in the networks and pathways among individuals, populations, and species, and know even less about the consequences of this variation for natural populations. Nevertheless, advances in systems/network biology, molecular biology, and 'omics' suggest a bright future for advancing our knowledge of how and when trade-offs are resolved in natural populations.

Acknowledgments

We would like to thank Karen G. Burnett, David S. Durica, Donald L. Mykles, and Jonathon Stillman for organizing and inviting us to participate in the Building Bridges from Genome to Phenome: Molecules, Methods, and Models Symposium and two anonymous reviewers for their useful and thoughtful comments.

Funding

This work was supported by a grant from National Science Foundation grant (IOS-1457383) to C.K.G and by a fellowship awarded to A.A.M that is included in National Science Foundation grant (DGE-1450032).

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