Review

The levels of analysis revisited

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The term *levels of analysis* has been used in several ways: to distinguish between ultimate and proximate levels, to categorize different kinds of research questions and to differentiate levels of reductionism. Because questions regarding ultimate function and proximate mechanisms are logically distinct, I suggest that distinguishing between these two levels is the best use of the term. Integrating across levels in research has potential risks, but many benefits. Consideration at one level can help generate novel hypotheses at the other, define categories of behaviour and set criteria that must be addressed. Taking an adaptationist stance thus strengthens research on proximate mechanisms. Similarly, it is critical for researchers studying adaptation and function to have detailed knowledge of proximate mechanisms that may constrain or modulate evolutionary processes. Despite the benefits of integrating across ultimate and proximate levels, failure to clearly identify levels of analysis, and whether or not hypotheses are exclusive alternatives, can create false debates. Such non-alternative hypotheses may occur between or within levels, and are not limited to integrative approaches. In this review, I survey different uses of the term *levels of analysis* and the benefits of integration, and highlight examples of false debate within and between levels. The best integrative biology reciprocally uses ultimate and proximate hypotheses to generate a more complete understanding of behaviour.

Keywords: integration; proximate; ultimate; levels of analysis

1. INTRODUCTION

Behaviour is studied from a variety of perspectives, ranging from social sciences, to behavioural ecology and animal behaviour, to cellular and molecular neuroscience. One of the greatest challenges of integrative biology is to synthesize ideas and information across such approaches to seek a more complete understanding. Integrative biologists are fortunate in that there is a rich and deep theoretical framework that can be used to aid such synthesis across different approaches. This theoretical framework is, of course, the grand unifying theory of all biology: evolution by natural selection.

The challenge of integration thus includes the challenge of considering evolution and adaptation when exploring the mechanisms of behaviour, and of considering mechanisms of behaviour when exploring evolution. Taking an evolutionary or adaptationist approach when studying mechanisms and developmental processes of behaviour has become increasingly popular in fields including behavioural ecology and evolutionary psychology, but has been extensively criticized (e.g. [1−4]). In this review, I explore the integration of adaptive function and causal mechanism. Central to this issue is seeking clarity about *levels of analysis* and *levels of reductionism*, and explicitly considering whether or not potentially

competing hypotheses are, or are not, mutually exclusive. First, I review varied concepts of the levels of analysis and attempts to synthesize or integrate between them. Second, I review several ways in which considering the ultimate function of behaviour can aid in exploring the mechanisms of behaviour. Finally, I make the point that clarity about whether or not competing hypotheses are mutually exclusive is required to eliminate false debate between and within levels of analysis. The examples I use below are biased to include studies on songbirds, in part because that is my own field of research, but also because birds are studied extensively from different perspectives across a range of disciplines and provide an excellent case study.

2. CAUSE AND FUNCTION

(a) *Levels of analysis*

Baker [5] distinguished between ultimate and proximate factors that regulate the timing of reproduction. Ultimate factors are those variables that determine offspring survival and thus reproductive success (e.g. food availability) and proximate factors are those variables that organisms actually use to time reproduction (e.g. the annual change in photoperiod). Mayr [6] refined and extended the use of these terms to highlight two kinds of questions biologists can ask about a phenomenon: 'what is the proximate causation?' (causes derived within the life of the organism) versus 'what is the ultimate causation?' (causes derived over evolutionary time). These two kinds of questions

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Table 1. Three uses of the term *levels of analysis*. I suggest that the distinction between ultimate and proximate levels is the best use of this term, though the other uses are now engrained in the literature.

levels of analysis		
ultimate/proximate	four questions	levels of reductionism
Mayr [6]:	Sherman [8] following Tinbergen [9]:	
ultimate (evolution and adaptation) proximate (mechanisms)	evolutionary history adaptive function development physiological mechanisms ?—cognitive/ psychological	ecological/ evolutionary organismal physiological cellular molecular

have become generally accepted as two levels of analysis. The proximate level (*how* questions) deals with mechanistic causes such as genetic, neural, hormonal, or cognitive/behavioural processes, whereas the ultimate level (*why* questions) deals with adaptive and evolutionary aspects. Ultimate 'causes' are causes only in the sense of an Aristotelian *Final Cause* and thus the ultimate and proximate levels have been summarized as function and cause, respectively [7].

In addition to the distinction between ultimate and proximate levels, the term *levels of analysis* has been used in other ways (table 1). A common example of this in animal behaviour is Tinbergen's [9] four questions of evolutionary history, adaptive function, causation and development. Tinbergen's framework is, justifiably, adopted in every major textbook in animal behaviour, and answers to each of these four kinds of questions are required for a full understanding of behaviour. Some authors, however, have equated these questions with the levels of analysis, and even proposed a fifth level for cognitive or psychological mechanisms (e.g. [8]). The term *levels of analysis* is additionally sometimes used to refer to levels of reductionism, such as behavioural, physiological, neural or molecular genetic levels of inquiry. I would argue that, given the distinct differences between mechanistic causes and adaptive functions—that is given they are distinct logical categories [10,11]—Mayr's division of ultimate and proximate levels is the most appropriate use of the term.

Table 1 illustrates that the term *levels of analysis* has been used in very different ways. However, it is important to note that the reference to levels need not imply a hierarchy or ranking of importance. Ultimate and proximate explanations are different explanations, but—despite claims by some—these two types of questions are not hierarchically related, and research at one level is not inherently superior to research at the other level [10].

Apart from providing theoretical scaffolding by which to organize different approaches to the study of behaviour, the levels of analysis are important in that they clarify non-overlapping explanations and

hypotheses [8]. For example, the statement that 'male birds sing in spring to attract mates' is not mutually exclusive with the statement that 'male birds sing in spring due to increased levels of circulating testosterone'. Of course, both of these hypotheses could be correct, or both could be incorrect. They are explanations at different levels of analysis; current adaptive function (ultimate) and physiological mechanism (proximate). Indeed, much fruitless debate could be avoided in some fields, such as evolutionary psychology, if opponents would acknowledge that both adaptive function and current socio-economic conditions can explain variation in human behaviour and that hypotheses at these two levels are not directly competitive. This issue is explored further in §2*b* below.

One of the key issues in debates centred on levels of analysis is whether information from one level of analysis can inform research at the other. Sherman [8] notes that non-overlapping explanations occur even within the proximate or ultimate levels of analysis. For example, hypotheses regarding the current adaptive utility (trait X is adaptive because of Y) and evolutionary history (trait X arose from a non-adaptive precursor) may be non-competing. I argue that mutually compatible explanatory hypotheses extend beyond traditional considerations of levels of analysis and do not only exist on different levels of analysis. An example of this is illustrated by the different hypotheses proposed to explain variation in immediate-early gene (IEG) expression in the auditory forebrain of songbirds (below). Whether or not two hypotheses are mutually exclusive is critical to preventing false debate either across, or within, levels of analysis.

(b) *Non-alternative hypotheses: immediate-early gene expression in songbird brains*

Measuring the expression of IEGs in the brain has become a standard method for assessing which neurons are repeatedly depolarized during exposure to a stimulus. For example, expression of the gene *c-fos* is increased in the medial preoptic area and the medial and cortical amygdala of mother rats following exposure to pups [12]. In songbirds, increased expression of the IEG *ZENK* (hereafter Zenk response) occurs in auditory forebrain regions including the caudomedial mesopallium (CMM) and caudomedial nidopallium (NCM) following exposure to conspecific vocalizations. CMM and NCM exhibit greater Zenk response following playback of conspecific song as opposed to other stimuli [13]. The importance of these brain regions for auditory processing of song has been corroborated by electrophysiology [14] and a lesion study [15]. The amount of Zenk response in the auditory forebrain has been shown to correlate well with variation in the nature of the song stimuli. For example, the Zenk response in the NCM is positively related to the complexity of playback songs in budgerigars, *Melopsittacus undulatus* [16] and the bout length of playback songs in European starlings, *Sturnus vulgaris* [17]. Moreover, in white-crowned sparrows (*Zonotrichia leucophrys*), the Zenk response is higher in birds exposed to preferred local dialect songs, and

correlates with the number of sexual displays emitted during song playback [18]. Thus, in a range of species, the Zenk response in auditory regions appears related to the biological salience of the song playback.

What accounts for this correlation between the nature of the song playback and the ensuing Zenk expression? One explanation is that increased activation of neurons in NCM and/or CMM reflects the auditory memories of songs learnt early in life. In support of this, the Zenk response to song playback is related to the accuracy of song learning in zebra finches (*Taeniopygia guttata*) [19,20]. Moreover, the Zenk response is higher in NCM and CMM following the playback of tutor song (compared with novel song) in young zebra finches in the process of song learning [21]. This differential Zenk response was absent in HVC (not an acronym, sometimes referred to as high vocal centre), a brain region critical for the production of learned song. Thus, growing evidence links the Zenk response in these caudal pallial regions with the memory of learned song that guides song production [22].

A different hypothesis used to explain variation in the Zenk response is that variation in IEG expression in the songbird auditory forebrain reflects attention mechanisms. Kruse *et al.* [23] found that changing the playback context increases Zenk expression in response to the same song. Merely switching the speaker location resulted in an increased Zenk response compared with the song playing from the same location. Similarly, male song sparrows (*Melospiza melodia*) show increased Zenk response in NCM when they are exposed to novel territorial songs as compared to when they are exposed to songs they had previously heard [24]. In female house finches (*Carpodacus mexicanus*) and song sparrows, there was no differential Zenk expression in response to playback of tutor versus non-tutor songs; however, in both species, Zenk expression was higher in isolate-reared females than in tutored females, consistent with the idea that the isolates were more attentive to songs when compared with tutored birds [25,26]. Combined, the results of the studies above are consistent with the idea that increased Zenk expression is related to increased perceptual processing via some form of attentional gating mechanism.

We thus have at least two testable hypotheses to explain variation in Zenk expression in the auditory forebrain. Hypothesis 1 is that increased Zenk response occurs because NCM and CMM provide the neural substrate for song memories. This hypothesis may or may not be correct for a number of reasons. For example, the locus of song memories may be elsewhere and the patterns of Zenk activation explained by other factors. Second, there may be no single locus of the song engram, and different aspects of song memories may be distributed over a range of perceptual and motor regions including NCM, CMM and the song-control regions such as HVC. Differentiating among these requires further experiments. Hypothesis 2 is that increased Zenk response reflects increased perceptual processing owing to an attentional gating mechanism. Similarly, this hypothesis may or may not be correct. The patterns described above are consistent with birds exhibiting variation in attention to song playback, but could result even in the absence of attention if, for example, the birds were asleep (see [21]). The important point is that these two hypotheses are on the same level of analysis (proximate) and address the same category of question (neural and cognitive mechanisms) but are still non-alternative explanatory hypotheses (*sensu* [8]). Both of these hypotheses could be correct in that increased Zenk could both reflect attentional processes *and* the locus of song memory; the two hypotheses are not mutually exclusive. Of course, future data could also prove both hypotheses incorrect.

This example highlights the importance of formulating clear hypotheses and determining whether or not different hypotheses are truly alternatives. Hypotheses at different levels of analysis are explicitly non-alternatives, but so too are many hypotheses within the same level. *Confusion over falsely competing hypotheses is not limited to hypotheses at different levels of analysis.* Some integrative and adaptationist approaches have been criticized for confusing levels of analysis (discussed in [1,8]). However, this problem is not limited to fields that attempt to integrate between proximate and ultimate levels, and occurs within fields that are exploring the same mechanisms in the same research domain. Careful attention needs to be paid to determine whether both hypotheses are mutually exclusive of each other or not, regardless of whether one is a causal and one a functional hypothesis, or both are functional hypotheses.

The example of IEG expression in the auditory forebrain highlights another point. Both of the above hypotheses can, and have, been criticized on the grounds that neither of them explains anything. Indeed, over the years, I have received several anonymous reviews pointing out that because it is unclear what is occurring at the cellular and molecular level, these hypotheses are void of mechanism. This category of comments reflects a failure to distinguish *levels of reductionism* (sometimes referred to as levels of analysis, table 1), and an assumption that analyses at lower levels of reductionism are inherently superior. Dennett [27] refers to reductionism that values lower levels and ignores complexities and theory at higher levels as 'greedy reductionism'. I refer to the view that research at lower levels of reductionism is inherently superior or more scientific as 'reductionist snobbery'. These views value physics over chemistry, and chemistry over biology. Greedy reductionism and reductionist snobbery are also present within biology, for example, when authors restrict the use of the work 'mechanism' to refer to cellular and molecular mechanisms and not behavioural or cognitive mechanisms, or explicitly value proximate explanations over ultimate [28]. Mechanisms, broadly speaking, include causal processes at the proximate level of analysis. These can include molecular, cellular or system level processes. The interaction of different organisms, different brain regions, different neurons, or different proteins can all be proximate mechanisms.

We do not fully understand how auditory processing in the songbird auditory forebrain results in increased expression of Zenk, nor do we know the downstream target genes in these brain regions and how their regulation affects activity in these brain areas. However, incomplete understanding at one level of reductionism

does not negate hypothesis testing at another level. Studies of cellular and molecular mechanisms, physiological mechanisms, developmental processes, and indeed cognitive and behavioural mechanisms are all at the proximate level of analysis.

(c) *Cause and function: integrating across ultimate and proximate levels of analysis*

Because explanations at different levels of analysis are not mutually exclusive alternatives, they cannot directly compete with each other. Indeed, there is almost unanimous consensus that we need explanations at both ultimate and proximate levels for a complete understanding of behaviour [2]. Some argue for boundaries between levels of analysis in order to eliminate confusion between them (e.g. [29]). Hogan [11] further points out that cause and function are logically distinct categories, and rejects Mayr's terminology of ultimate 'causation'. Because adaptations are the outcome of selection, it is teleological to argue that adaptive function can, in any sense, be an ultimate cause of behaviour. More to the point, Hogan [11] argues that function can tell us nothing about proximate causation. Similarly, Bolhuis [2] argues that, at most, functional considerations can provide only clues as to potential mechanisms.

Despite the clear distinction between proximate and ultimate levels of analysis, however, approaches that integrate across these levels are thriving. Behavioural ecology, traditionally focused on ultimate questions, has become more and more focused on proximate mechanisms including immunology, endocrinology, neurobiology and development. Such approaches use information from the proximate level of analysis to help formulate and revise hypotheses at the ultimate level. Indeed, many argue that a failure to integrate across levels is likely to lead to erroneous interpretations [30], and that data and interpretations drawn from one level can inform data and interpretations at the other. McNamara & Houston [31] argue that it is necessary to integrate mechanisms when exploring the evolution of behaviour.

Not all agree that integration across levels of analysis is a useful research strategy, however (e.g. [1]), and suggest that instead research at the proximate level should focus on naturally occurring behaviours, but without presuppositions as concerning the evolutionary or functional significance of them. I disagree. Taking an adaptationist stance—assuming that neural and cognitive mechanisms have function, and have been shaped by natural selection—does more than providing clues as to their mechanisms. Assuming adaptive function or design (the intentional or adaptationist stance) is an efficient and sensible way to figure out how something works [27,32]. In reverse engineering something, an adaptationist stance (assuming design) constrains and directs lines of inquiry from vast to those best suited to the data [27,32]. The discovery of *how* the heart acts as a pump, for example, was no doubt aided by the assumption that the heart has a (adaptive) function: that of, well, a pump. Similarly, the study of mechanisms of behaviour can benefit

from the adaptationist stance. A research programme aimed at understanding mechanisms of behaviour that truly and entirely ignores function becomes a Brownian random walk at the proximate level.

It is important to note, however, that an adaptationist stance does not imply Panglossian hyper-adaptationism (*sensu* [33]). Assuming an adaptive function as a way to develop and test hypotheses is not synonymous with accepting such hypotheses despite evidence to the contrary. Particular neural or behavioural mechanisms cannot be automatically assumed, just because a behaviour has a particular adaptive function. An integrative approach uses considerations of function and evolutionary history to generate hypotheses as to proximate mechanisms, and then proceeds to test, and possibly to reject, these hypotheses [34].

Adaptationist integrative biologists use functional considerations to interpret data and generate hypotheses. Sherry [35,36] highlights several ways that a consideration of adaptive function can aid in the study of mechanisms. First, function can provide guidance in developing hypothesis at the proximate level (§3*a*). Second, adaptive function can define the categories of behaviour that are further explored at the proximate level (§3*b*). Third, consideration of function can set criteria that proximate explanations must satisfy (§3*c*). In addition to these benefits, an integrative approach has at least one other major strength. Not only does a consideration of adaptive function help guide research and interpretation of proximate mechanisms, but data and interpretations of proximate mechanisms often reciprocally guide and refine functional and evolutionary hypotheses (§3*d*).

3. THE ADAPTATIONIST INTEGRATIVE APPROACH

(a) *Generating hypotheses*

The power of the adaptationist stance to generate novel hypotheses is a major strength of an adaptationist integrative approach, such as neuroecology or behavioural ecology. Sherry [35] documents several examples including olfactory learning in rodents resulting in disassortative mating at the major histocompatibility complex [37] and the use of hippocampus-dependent spatial memory to retrieve cached food by chickadees [38,39]. The first of these examples arose from evolutionary theory on inclusive fitness [40], which raises the proximate question: how do animals recognize their relatives? The second example arose from a functional consideration that food-storing birds increase overwinter survival through caching, which raises the question: how do birds retrieve cached food?

A third example is sex ratio manipulation. Following long-standing theory that sex ratios should be maintained at 1 : 1 [41], Trivers & Willard [42] predicted that parents should be able to adjust offspring sex ratio depending on their condition. This differential sex-allocation theory has been supported by numerous studies, and there is good evidence that this can even apply to primary sex ratios in vertebrates. For example, collared flycatcher (*Ficedula albicollis*) females appear able to adjust the sex ratio of eggs ovulated in response to the phenotype of their mate [43].

This raises the novel proximate question: how do birds adjust the primary sex ratio of their eggs? Recent evidence suggests that hormone levels of the mother may play a role. For example, high progesterone during meiosis can bias sex ratios towards overproduction of females [44]. Other hormones may affect primary sex ratios as well, and research is ongoing (reviewed in [45]). Much study remains to work out the molecular mechanisms of this remarkable process. However, it is unlikely that any of these research groups would have undertaken the detailed and expensive mechanistic studies to explore how birds manipulate primary sex ratio without previous evidence that birds do indeed manipulate primary sex ratio in the wild. These field observations were in turn motivated by predictions from sex-allocation theory that assumed adaptive function. Consideration of adaptive function provides motivation and direction to explore novel proximate mechanisms.

Of course, consideration of adaptive function sometimes generates proximate hypotheses that are not supported, or are not good ideas to begin with. For example, one might predict that since, in many songbird species, males learn to sing and females do not, males might have been selected to be better auditory learners than females. As noted by Bolhuis & Macphail [1], there is no evidence for this hypothesis. Of course, one might also predict the opposite. Since, in many species, females are the primary receivers of song, might they be superior to males in auditory perception or auditory learning? Evidence suggests that this may be the case in some species. Although data are scant [46], some evidence indicates that female Bengalese finches (*Lonchuria striata*) are more sensitive to changes in song stimuli than males, as assessed by changes in heart rate [47]. Similarly, female red-winged blackbirds (*Agelaius phoeniceus*) appear to be more perceptive of songs than are males [48]. Whether or not a hypothesis about proximate mechanisms that was derived from evolutionary predictions is supported by the data, or was a good idea to begin with, is unrelated to the fact the hypothesis was based on ideas from the ultimate level of analysis.

As one further example of the importance of using information on adaptive function to explore mechanism I return to the issue of which regions in the songbird brain underlie song perception and auditory song memory. Much of the early research on the neural bases of song perception and auditory memories focused on the song-control system, brain regions demonstrated to be important for song production and acquisition [22]. Current evidence suggests that this may not be the case, and instead good candidates for the regions important for perception and song memories include auditory regions, such as CMM and NCM. Considering function, however, directly influenced the discovery that these areas are important for song perception. For example, the consideration that song functions in female attraction led to the hypothesis that, because female zebra finches have a tiny HVC compared with males [49], it would be unlikely that the song region HVC is important for song perception. That is, if HVC is critical for song perception, it should not be so small in female zebra finches. As may be predicted, HVC lesions did not disrupt song preferences in female zebra finches, but CMM lesions did [15]. Earlier work on marsh wrens (*Cistothorus palustris*) similarly suggested that HVC might not be the primary brain region underlying song perception and song memories. Males of the western subspecies sing more complex songs and have a larger HVC than males of the eastern subspecies [50]. However, HVC size differences between these subspecies do not reflect song-learning experience [51], nor are there differences between the subspecies in HVC size in females [52]. These studies took an adaptationist, integrative approach and assessed whether variation in HVC size might reflect differences in song learning or song perception. The results did not support this idea, and the search for the neural substrates of song perception continued.

If birds are adapted to process species-specific songs differently from other sounds, they should possess brain regions that receive auditory input and respond specifically to song more strongly than other sounds. This is exactly what was found with the discovery of the connectivity of CMM and NCM to other auditory regions and the IEG response of these regions to song playback [13,53,54]. As noted in §2b, above, there is growing evidence that these regions are critical for song perception in many species, and may also function as the neural basis of song memories. It is important to note, however, that these neural regions important for specialized auditory processing of song, and which may also serve in storing auditory memories of song, were discovered by considering clues suggested by adaptive function, *not* by considering the behaviour in the absence of presuppositions concerning their evolutionary or functional significance. It is too early to entirely rule out a role for the song-control brain regions as important for song perception and memories, as correlations and lesion data from at least canaries (*Serinus canaria*) do support this [55–58]. Further work is clearly required to better understand how the song-control system and specialized auditory regions such as CMM and NCM coordinate song production and learning, song memories and song perception. These efforts will benefit from comparative approaches that test whether neural differences reflect behavioural differences among taxa.

(b) *Defining categories*

Besides generating novel hypotheses, adaptive function defines the categories of behaviour that we study. Function defines fields. Indeed, physiological systems are often named for what they do: circulatory system, immune system, digestive system. Though not commonly thought of in this way, these fields reflect the functional organization of physiology, shaped through natural selection in response to the competition for survival and reproduction. The function of the digestive system, though sometimes considered in the absence of evolution, is indeed an adaptive function. Individuals better able to extract required energy and nutrients from their food will outcompete those less able to do so.

In behaviour, considering adaptive function similarly defines clusters of behaviour that are studied together: communication, foraging, kin recognition. Moreover, considering adaptive function can help resolve categorization of behaviours in ways that may assist understanding of mechanistic causes. For example, maternal aggression in rodents is phenotypically similar to other forms of aggression, but its endocrine regulation involves changes in hormones associated with pregnancy and maternal behaviour, such as oestrogen and progesterone [59]. Thus, considering the function of maternal behaviour can assist in the exploration of mechanism. That function defines categories of behaviour that are studied together is more or less self-evident. However, these categories can also guide the studies of proximate mechanism. For example, pre-existing neuroendocrine mechanisms can be co-opted through evolution to control different behaviours with similar function [60]. The gonadal steroids (e.g. testosterone, oestradiol) that regulate gamete production later evolved to control sexual behaviour. The peptide hormone prolactin triggers brooding and crop milk production in male ring doves (*Streptopilia risoria*; [61]) and is associated with paternal behaviour in California mice (*Peromyscus californicus* [62]). In this case, the common adaptive function (paternal care) may be underpinned by similar proximate mechanisms (prolactin).

However, it is critical to also note that very different mechanisms can give rise to similar outcomes. For example, evolution can result in changes in the hormone-dependent behaviours that may not be initially predicted by ecological or evolutionary perspectives. Testosterone may be critical for the expression of sexual behaviour in one species, such as laboratory rats [63], but not in another, such as red-sided garter snakes (*Thamnophis sirtalis* [64]). Similarly, work in evolutionary developmental biology has shown gene expression underlying traits with the same adaptive function may, or may not, be the same. For example, eyes in mammals and insects appear to have evolved independently, but their development is influenced by the regulatory gene *Pax6* in both taxa [65]. Such deeply homologous traits that have different morphology may develop through expression of the same genes [66]. In this case, the adaptive function (vision) may be implemented by different non-homologous morphological structures (eyes) in different taxa, that are, in turn, dependent on developmental expression of (deeply) homologous regulatory genes.

Adaptive function defines categories of behaviour. The mechanisms within these categories may or may not be similar across taxa. Thus, functional categories may or may not reflect categories of mechanisms. However, considering adaptive function provides a useful framework to explore mechanisms within a category to determine where they differ and where they are the same.

(c) Setting criteria

Considering function can also set criteria that hypotheses about proximate mechanisms must satisfy if they are to have any explanatory power [35]. Part of the reason for this involves design constraints [27]. The number of shapes that can be used to create a useful wheel are limited. Thus, hypotheses about how an object can be moved using rollers are limited in scope. Similarly, there are only so many ways that an object can be spatially located by sound, or that the earth's magnetic field can be used for navigation [35]. Hypotheses regarding the neural mechanisms of sound localization by vertebrates must deal with inter-aural time, intensity and phase differences. These are the mechanistic bases by which the problem can be solved. Hypotheses regarding the sensory and neural mechanisms of magnetic compasses must deal with polarity, strength and inclination of the earth's magnetic field. These are the kinds of information that could theoretically be used by a magnetic compass. It is the assumption that natural selection has designed sensory systems with a function that allows us to define the problems that neurosensory systems must solve [35]. Thus, consideration of the adaptive function of the system (e.g. sound localization) sets criteria that any potential mechanistic hypotheses must satisfy.

Although the adaptive function of behaviour sets criteria that must be met, the discovery of proximate mechanisms may take unexpected turns. Factors that might be expected to be important based on design criteria may turn out to not be. For example, the regulation of seasonal reproduction must rely on environmental cues that change seasonally which can affect internal timing mechanisms. Photoperiod is one such environmental cue, and it might be predicted that declining day-length would act as a proximate signal to terminate reproduction in spring/summer breeders. However, in many species of songbirds, the declining length of daylight in late summer has nothing to do with the termination of seasonal reproduction. Rather, exposure to long days in early spring, by mechanisms not fully understood, results in the eventual onset of photorefractoriness and termination of reproduction [67,68]. Similarly, design considerations constrain the types of cues that could be used by a magnetic compass, and strong evidence indicated early on that migratory birds use inclination [69]. However, the more recent discovery that cryptochromes may be the molecules that underlie birds' light-dependent magnetic compass did not simply result from consideration of function, and depended on the input from physics theory [70]. Thus, considering adaptive function can set design criteria and inform exploration of proximate mechanisms, but is clearly not sufficient in and of itself for the discovery of such mechanisms.

(d) Proximate–ultimate reciprocity

There is at least one other benefit of considering adaptive function when exploring proximate mechanisms. This occurs when data on proximate mechanisms are then used to refine and direct hypotheses at the ultimate level of analysis. Such reciprocity between levels of analysis can be the hallmark of the best integrative biology. Here, I review several examples where

consideration of mechanisms has guided the generation of hypotheses regarding evolution and function.

Fluctuating asymmetry provides an example where a proximate mechanism resulted in a large number of functional hypotheses. Fluctuating asymmetries are random deviations from perfect symmetry in bilaterally symmetric organisms, and individuals with greater asymmetry are presumed to have lower ability to cope with environmental stressors during development (often referred to as developmental stability [71]). Consideration of mechanisms of development resulted in wide interest in behavioural ecology in the functional significance of fluctuating asymmetry in visual signals in a range of taxa [72,73]. In many species, symmetric individuals are preferred mates when compared with more asymmetric individuals [74]. In turn, considering the function of preferences for symmetry generated novel proximate hypotheses. At the ultimate level, individuals may be selected to prefer symmetrical mates because such mates may pass on heritable traits that allow stable development. Thus, perhaps symmetrical features are signals that convey information about an individual's developmental stability to potential mates. This ultimate hypothesis then begs the proximate question: can individuals perceptually detect fluctuating asymmetry? In a series of studies, Swaddle [75] and Swaddle *et al.* [76] have shown that the ability of birds to detect asymmetry in visual features is limited. Birds can detect asymmetries in visual displays, but not the small differences such as observed in asymmetrical morphological traits. These new data on proximate mechanism must now be accounted for and lead to the revision of ultimate hypotheses. If asymmetry is not detected, how can it be a signal? Perhaps asymmetry is correlated with other traits that are detectable and do act as signals. Studies of fluctuating asymmetry have had their share of methodological and theoretical challenges [73,77]; however, the reciprocal flow of data and interpretations have moved the field forward.

A second example of proximate and ultimate reciprocity involves sex differences in song-control regions of the songbird brain. Shortly after the discovery of the song-control system, Nottebohm & Arnold [49] found that brain regions such as HVC exhibited one of the largest anatomical neural sex differences observed in vertebrates. In zebra finches, females cannot sing, and male HVC is an order of magnitude larger than that in females. In canaries, however, where females can sometimes sing (but less than males), the sex difference was smaller. This proximate observation led Brenowitz & Arnold [78] to test the ultimate hypothesis that perhaps sex differences in the brain co-evolved with sex differences in behaviour. In a study of tropical wrens where males and females sing equally, they found no sex differences in the size of HVC. Since that time, the story has become more complex, and the interplay between proximate mechanism and function and evolutionary history has continued. In some species where females sing equal to males, sex differences in HVC are still observed [79–81]. However, comparative analyses do demonstrate a correlation between sex differences in behaviour and in the brain [82], but it is not a simple correlation. Sex differences in HVC are affected by phylogeny, and exhibit a persistent male bias [83]. Indeed, a recent study of a species where females sing more than males still found a male-biased HVC size [84]. In another species, where males and females have identical songs, males have a larger HVC, but females' HVC has much higher expression of synapse-related genes [85]. Such studies raise many further questions at both proximate and ultimate levels. What was the ancestral condition; have sex differences increased or decreased over evolutionary time? In species where females sing equally to males, but have a smaller HVC, how do they accomplish this? Do they use alternative neural mechanisms than males to learn and produce song? Do they incur any developmental trade-offs by using such mechanisms? Understanding of the evolution of the sex differences in the song-control system will require better understanding of the development of these differences. Although initially considered a model for the organizational effects of gonadal steroids, currently these sex differences are thought to emerge from sex differences in the genome [86]. Further work is required to determine both the evolution and mechanisms of sex differences in the song-control regions of the brain, but this will be aided by consideration of both ultimate and proximate levels of analysis.

Finally, studies of proximate mechanisms can influence studies at the ultimate level by highlighting constraints that need consideration. Animals are not infinitely flexible in their capacity to respond to the environment. A recent review has called for a systematic study of the evolution of mechanisms ('evo-mecho'), with a focus on simple mechanisms that work well in diverse environments as opposed to complex mechanisms that work well in simple environments [31]. It is unclear to what extent this call will be heeded, but it is important to note that there is growing consensus that studies of behaviour at the ultimate level of analysis cannot ignore proximate mechanisms.

4. CONCLUSION

In this review, I have highlighted different uses of the term levels of analysis, and explored the benefits of using an integrative approach that uses an understanding of adaptive function to guide exploration of proximate mechanisms. This endeavour of integration is not without risk, however. It is critical to thoroughly evaluate whether or not two hypotheses are mutually exclusive. By definition, hypotheses at the ultimate and proximate levels of analysis are not mutually exclusive, but it is often ignored that presumably competing hypotheses within a level of analysis, or at different levels of reductionism, may also be non-exclusive. That is, two hypotheses may be non-exclusive even if they are at exactly the same level of analysis and level of reductionism. Much false debate could be avoided by careful consideration of whether or not alternative hypotheses are or are not mutually exclusive alternatives.

Because ultimate and proximate levels do not directly compete, however, does not mean they should have nothing to do with each other. An

adaptationist, integrative approach uses knowledge of one level to guide research at the other. Explanations at these two levels must at least be concordant, even if they do not directly compete. Failure to consider ultimate hypotheses when conducting research on proximate mechanism can result in 'greedy reductionism'. Similarly, researchers at the ultimate level need to be careful to not underestimate the complexities of proximate mechanisms of the behaviours under their study. Integrating across levels of analysis is tricky business. Researchers often have primary expertise and training in one level or the other. They must thus tread carefully when in each other's domain. A common charge is that evolutionary ecologists have too simple a view of proximate mechanisms, or that cellular and molecular biologists have too simple a view of behaviour and ecology. Indeed, I suspect that many of the critiques of integrative approaches stem from researchers in one field failing to address or appreciate complexities in another. Integration across levels of analysis thus requires detailed knowledge of other research domains, and is thus often best conducted via collaborations.

A complete understanding of behaviour, or any biological phenomenon, requires explanations of both cause and function. Achieving these explanations is best done in an integrative fashion. Consideration of function can guide research into mechanisms in many ways. Similarly, research into adaptive function can be stimulated and guided by a detailed appreciation of mechanisms. Although there is risk of confusion, careful consideration of one level of analysis can benefit research at the other.

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