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Studying adaptation at the invisible scale

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In order to understand adaptation by natural selection, it is necessary to observe organisms in their natural habitat. For this reason, the field of behavioral ecology, which specializes in testing adaptive explanations for biological observations, is dominated by research on larger multicellular animals such as insects, mammals, fish, and birds. The vast majority of modern life sciences, however, is concerned with the study of cells, genes, and molecules, which are often impossible to observe directly in nature. This severely compromises our ability to complement mechanistic understand of traits of interest with adaptive understanding. This matters because only the theory of adaptation can provide an explanation for *why* biology operates in the way that it does, why it varies across individuals and species, a formal tool for making predictions about the future. The good news is that technological advances are creating new opportunities for understanding cellular and subcellular traits as the products of natural selection.

behavioural ecology | adaptation | genomics

> *"..for life achieves its summit when it does to the uttermost that which it was equipped to do.", White Fang,* Jack London, 1906

What Is an Adaptive Explanation?

Pioneer of molecular biology, Max Delbrück (1906–1981), and author of a foundational study of genetic structure and mutation, was a physicist, not a biologist. On the challenge of transferring his interests to the living, he wrote: "*there are no 'absolute phenomena' in biology. Everything is time-bound and space-bound. The animal or plant or micro-organism he is working with is but a link in an evolutionary chain of changing forms, none of which has any permanent validity*" (1). Even at the very dawn of modern molecular biology, it was obvious (even to a physicist) that if you want to understand a living thing, or part of a living thing, it is necessary to consider not just how it works as a piece of machinery, but as the product of an on-going dynamic process. No sensible biologist would disagree (2).

The only way to answer *why* questions about biology is to apply the theory of adaptation by natural selection (Box 1). This requires an understanding of selection and, therefore, the environment in which an organism lives (3, 4). For example, if we explain the shape of a bird's bill as an adaptation to exploit large seeds, we can explain variation between individuals and/or taxonomic groups, and we can make predictions about how bill shape would evolve in the future if seed availability changes. It can also explain why gene expression is facultative or constitutive; or why genetic architecture is simple or complex. A mechanistic understanding of how a bill is formed cannot do this and neither can a phylogenetic tree (Box 2).

Despite the power of Darwin's theory of natural selection, researchers in the life sciences have rather left it behind in their quest to understand life at ever smaller scales (Box 3). Simultaneously, the field of behavioral ecology has developed expertise in generating hypotheses about adaptation and testing them in a rigorous manner (3, 10). The primacy of studying organisms in their natural environment to understand adaptation has been demonstrated again and again (4, 13). For this reason, behavioral ecology is dominated by research on larger, multicellular organisms that can be directly observed. The result is a divide in the life sciences between mechanistic studies on tiny things and functional studies on things that we can observe by eye.

Views on how much this matters may vary but only adaptation can provide an explanation for function, variation, and make predictions about how a trait will change in the future. What is also undeniable is that the tools we need to address this imbalance are increasingly available, and opportunities are out there to make startling, new discoveries about "why things are the way they are" down to the molecular scale. There is work to be done both by molecular biologists thinking about adaptation and by behavioral ecologists switching to invisible study systems. As usual, the most exciting progress will likely be made by biologists working together across fields.

In this review, we take a tour around some of these new opportunities to study adaptation at the invisible scale, thanks to technological advances in genomics and visualization. We highlight research that has already been successful in moving adaptive explanations from speculation to rigorous hypothesis-testing. These studies provide examples of the step-change advances we can make in our understanding of molecular and cellular biology as a result of this approach. Finally, we consider some of the conceptual as

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Box 1.

A primer on generating testable evolutionary hypotheses:

Darwin pointed out that natural selection is an "improving process" that leads to organisms becoming better adapted to their environments across generations (5). But if we want to make testable hypotheses, then we need to know exactly what natural selection improves.

Natural selection favors genes that are more effective at transmitting themselves to future generations. Consequently, organisms will appear as if they designed to maximize their genetic contribution to future generations, or "fitness" (6). Fitness can be complicated, but luckily, we have two approximations that make things simple. In many cases, fitness can be approximated as the number of offspring that an individual will produce in their lifetime. In practice, we often must use some proxy for lifetime reproductive success, such as the efficiency of a process or the rate of energy intake. At the invisible scale, we might examine the efficiency with which an enzyme can break down a nutrient or the efficiency with which ribosomes convert resources into proteins.

But genes cannot just increase their frequency in the next generation by influencing the reproductive success of the individual that they are in. They can also influence the reproductive success of related individuals (kin) that are likely to be carrying that gene. In situations where there are interactions between relatives, we can use another simple approximation, that natural selection will lead to organisms maximizing their "inclusive fitness" (7).

Inclusive fitness sounds complicated, but it just sums the consequences of a behavior or trait for the lifetime reproductive success of the individual performing it, and their relatives, with the consequences for relatives weighted by genetic relatedness. Genetic relatedness (R) is a statistical measure of genetic similarity between two individuals, which measures the extent to which the actor helping another individual is like the actor helping itself (8). Interactions with both relatives and nonrelatives are important at the invisible scale. For example, the clonal growth of single-celled organisms often means they will be neighboring and interacting with clonemates (9). Inclusive fitness tells us how to weight those interactions.

The fitness maximization approach facilitates the generation of testable hypotheses about adaptation because it tells us that we can take an economic approach and focus on the costs and benefits of traits. It takes evolutionary theory based on gene dynamics, which is how natural selection operates, and translates it into a theory about adaptation at the individual level, in ways that can be experimentally tested. We can think of any organism, or any part of an organism, as a "maximizing agent" that is trying to maximize its fitness. This approach has revolutionized our understanding of large multicellular organisms over the last 50 y, and the stage is set for a similar revolution at the invisible level (10, 11).

well as practical challenges involved in studying adaptation at the invisible scale, and also some of the problems that arise in efforts to integrate evolutionary theory more effectively to the wider life sciences. But first, let us consider the primacy of observation across biology.

Watching and Wondering

Ever since microscopes were invented, the biological study of tiny things has been driven by, and has driven, technological innovation. Observation of the previously invisible has advanced though molecular techniques, PCR, western blots; through genomics and advances in cell biology: genetic modification and in situ hybridization. These techniques have led to a revolution in our understanding of the natural world and how life works. They are less useful, however, at answering questions about *why* living things do the things they do and that is because answering these questions requires a living thing to be observed in the environment in which it has evolved. Darwin understood this well, and researchers that study adaptation today, still usually rely on being able to watch their study organisms in their natural habitat (4, 13).

Behavioral ecologists have also made use of the technological revolution in biology to make observations of behavioral and structural traits that are invisible to the naked eye—the parentage of a clutch of eggs (14), a magnetic compass in the mind of a pigeon (15), antimicrobials secreted by a burying beetle (16). The difference is that behavioral ecologists are asking questions about function—why do these traits enhance fitness in the natural environment? Why has this strategy outcompeted alternatives? How do we explain variation in this trait between individuals or between species?

Niko Tinbergen, one of the founders of behavioral ecology called this approach to studying adaptation, "watching and wondering" (Box 2) (12). Inspired by watching birds on the Dutch sand dunes, he made meticulous notes and sketches of what he saw; formulated hypotheses about the adaptive interpretation of his observations and designed ingenious experiments with which to test these hypotheses (17). He provided a conceptual framework that organizes explanations for biological observations into four questions (Box 2) (12). To Tinbergen, it was clear that mechanistic and evolutionary explanations are complementary and necessary. He also provides clarity about the distinction between studying evolutionary change and adaptive function, both necessary but distinct components of what are commonly referred to as "ultimate explanations." Mechanism and ontogeny (development) comprise the two aspects of a "proximate explanation" (2, 12). This Tinbergian approach has revolutionized the biological study of large, visible organisms since the 1970s (3, 10). But it has yet to have the same impact on the study of life at the invisible scale.

Box 2.

	Category	Explanation	Example
Q1	**Causation (Mechanism)**	What are the immediate biological mechanisms underlying the behavior?	Hormones triggering bird song in the spring.
Q2	**Development (Ontogeny)**	How does the behavior develop during the lifetime of an organism?	A bird learning its song by imitating adults.
Q3	**Function (Adaptation)**	What is the evolutionary purpose or benefit of the behavior?	Birdsong attracting mates to increase reproductive success.
Q4	**Evolution (Phylogeny)**	How did the behavior evolve over the history of the species?	Birdsong originating in an ancestor common to songbirds.

Tinbergen's four questions:

Tinbergen maintained that in order to properly understand a trait, either behavioral or structural, it is necessary to ask four questions about its causation; "Tinbergen's Four Questions" as listed in the table above (3, 12). The four questions are divided into two questions about proximate causation (mechanistic) and two questions about ultimate causation (evolutionary). When we apply this framework to the example of the coloration on bird eggs, it is easy to see how incomplete our understanding would be if we focused on just mechanism (we would understand splodges but not what they were for) or just evolution (we would understand that they were an adaptation for improved camouflage but have no idea how they were produced). It also clarifies the distinction between studying evolutionary change (Q4) and explaining that change in terms of its adaptive function (Q3). Technological advances have led to an explosion in genomic data, creating new opportunities to map evolutionary change in ever-increasing amounts of volume and detail. But tracking how an allele has spread through a population only describes evolutionary change, it does not explain why it has increased in frequency. Similarly, identifying conserved protein residues across a phylogeny may reveal diversity but it does not explain that diversity: Why a protein of a particular structure is present in some lineages but not others. The only way to answer these *why* questions is to apply the theory of adaptation by natural selection. Image credit: Wikimedia Commons/Rob Mieremet (ANEFO), licensed under CC0 1.0. -SG.

Why Does Understanding Adaptation Matter at the Invisible Scale?

Asking questions about adaptation at the invisible scale will bring the same insights into molecular and cell biologists that it has brought to biologists studying animals, plants, and fungi (*SI Appendix*, Table S1). These can be divided into three categories:

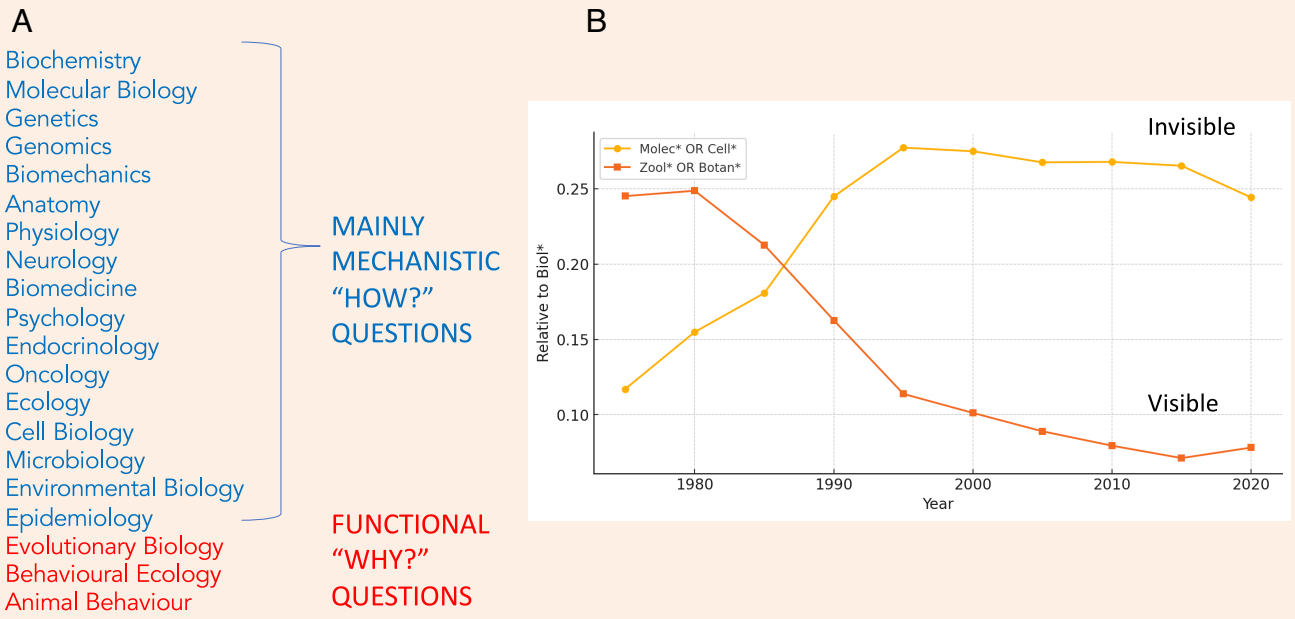
1. Function: An adaptative explanation is the answer to "why?" to complement an understanding of "how." This is an explanation for why a biological structure, process, or behavior exists in the first place. The ultimate answer to this question comes, and can only come, from the application of Darwin's theory of natural selection. It does not come from the study of evolutionary change, a phylogeny or a SNP analysis (Box 2).
2. Variation: Characterizing variation is not the same as explaining variation but it is a necessary first step. Biologists have revealed the fascinating and staggering diversity in which life operates at molecular and cellular scales and the opportunities are now wide-open to explain that diversity, Only by studying traits as the product of natural selection can we achieve this (Box 2, Fig. 1, and *SI Appendix*, Table S1).
3. Prediction: An adaptive approach generates formally justified, a priori predictions. If we understand a trait as the product of natural selection, we have the power to make predictions about what populations of organisms might behave in environments they have never encountered. This tool is crucial for facing many of today's global challenges, including climate change, infectious disease, antibiotic resistance, and sustainable development (10, 21).

The Challenges

If a biologist studying the interaction of a protein with a receptor on the surface of a cell membrane wanted to apply Tinbergen's approach, how would she go about this? How can we understand the selective forces acting on a membrane receptor? Can we understand individual variation in the operation of this receptor? Can we even measure variation, at all?

Microbiology is based on the study of cells we refer to as "isolates," precisely because we *isolate* them from the environment in which they would naturally live. We store isolate collections in freezers, in monoculture, and grow them in rich liquid media, in sterile glass tubes; far removed from where they might have evolved to live: in the sludge at the bottom of a pond, or inside someone's intestines. Worse, even if we were to go out to find bacteria living wild and free, like Tinbergen in the sand dunes, looking for birds, what could we learn from looking at a square foot of soil about the selection pressures facing the billions of invisible organisms in there; organisms that do not even experience the laws of physics—gravity, electromagnetism, hydrostatic tension—in the same way that we experience them?

Box 3.



A mechanistic age in the life sciences:

Biology moves into the invisible realm *A*: A survey of how the names of biology departments have changed across the life sciences reveals how dominant the focus on mechanism has become. Even fields which are often associated with the study of evolution, such as genetics and genomics, are often concerned with how evolutionary change takes place, rather than why (Box 2). B: The switch from visible to invisible life is evident from changes to how institutions in the life sciences are named. If you studied biology 50 y ago, you would likely have belonged to a Botany or a Zoology department. Those departments have almost disappeared—there is only one "Department of Zoology" left in the United Kingdom—most having switched to a name that requires an acronym. This trend in the naming of departments reflects a deeper and more fundamental trend in the life sciences over a very short space of time—the trend toward the study of "invisible things": life that cannot be observed with the naked eye. The proportion of papers with "Molec* OR Cell*" in the address field of a Web of Science search has risen in almost an exact mirror image to the decline of departments with "Zool* or Botan*" in the address field (both scaled to the numbers of papers with Biol* in the title).

Studying adaptation at the scale of cells and genes, can also come with conceptual challenges of identifying the target of selection—or to put it another way, can we safely treat the individual organism as a fitness maximizing agent (22, 23)? This is key for making predictions about how we expect a trait to evolve. Genomic imprinting, for example, is a molecular-level trait which is favored by selection acting on the parents of the organism in which the trait is expressed (24). The phenotype of these genes only makes adaptive sense in the light of its origin (the male or female parent), not the individual in which it is expressed (the embryo). Similarly, cancerous tumors can be understood as the result of selection to proliferate and compete for space and resources more effectively. The phenotype of these genes only makes adaptive sense at the level of a single cell linage, not the organism as a whole (25, 26).

These examples emphasize why a biologist who wants to understand adaptation might prefer to work on an animal she can visit and watch through a pair of binoculars. Is it even possible to understand ultimate causation of traits that have evolved in a world invisible and inaccessible to us?

New Opportunities for Studying Adaptation at the Invisible Scale

The good news is that opportunities to answer ultimate questions about invisible things are increasingly available. It may even be the case that we are on the brink of a Tinbergian revolution in the life sciences, when the study of molecules and cells starts to explore questions beyond mechanism. One of the advantages of studying evolution in single-celled organisms with relatively short generation times is that we can track the adaptive process in real time by monitoring changes in allele frequencies in populations living within an individual host (27) or at the global scale (28). In traits with strong fitness effects, such as antibiotic resistance, we can be reasonably confident about making functional hypotheses. But adaptation is rarely so clear-cut, and it may not be straightforward to sample populations to test adaptive hypotheses, for example, in pathogens. In the following section, we provide an introduction to some strategies that can overcome these difficulties to test hypotheses about adaptation and identify selection pressures.

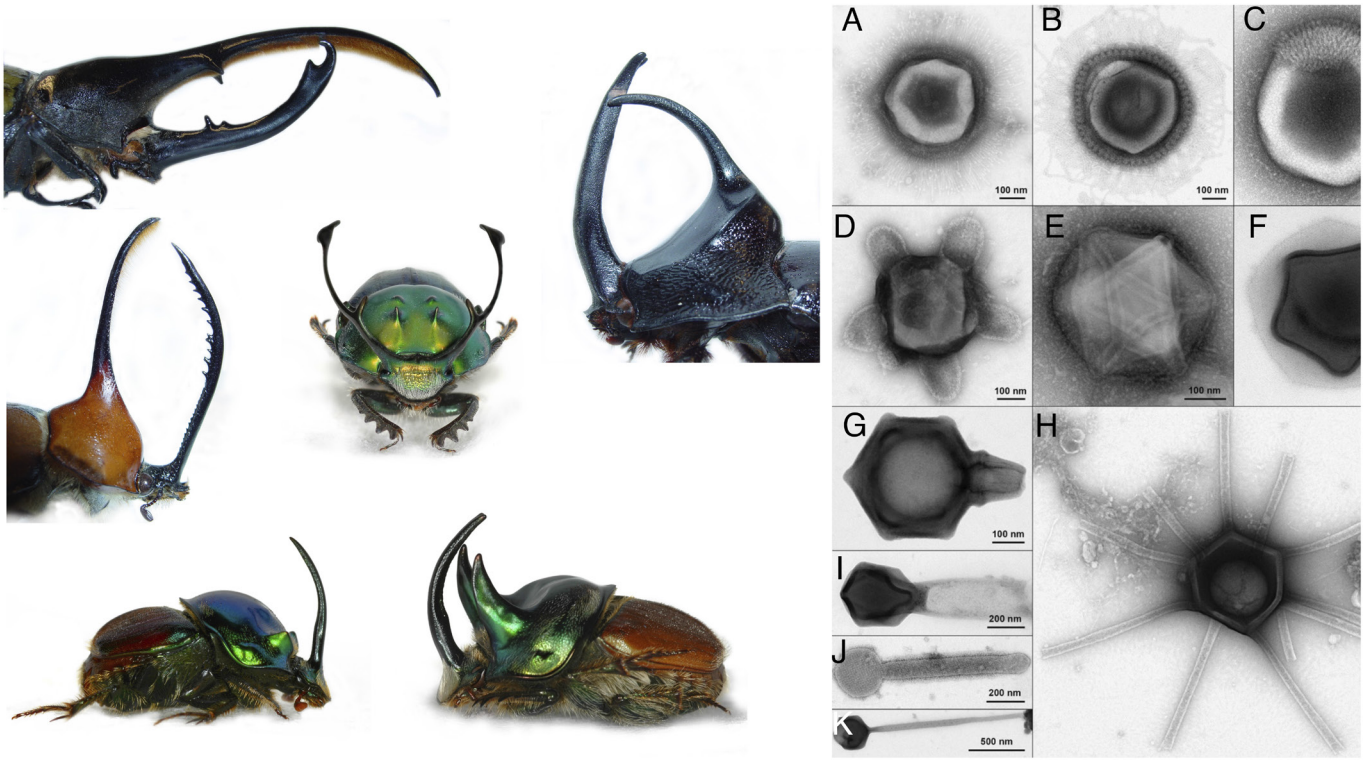


Fig. 1. The mystery of the scarabs (and the giant viruses): Darwin was one of the first to note a particular propensity for diversity in male scarab beetles (18) (*Left*-hand panel). The propensity and the diversity have now been explained by Emlen et al.: horns function as weapons for fighting competitor males for access to females. Variation in form is due to variation in use: some species fight in tunnels (short, stubby horns work best) and others fight in the open where a "lance" style weapon is more effective (19). The propensity for diversity can also now be explained by a particularly plastic developmental pathway. This research provides a great example of the progress that can only be made by linking adaptation and mechanism together, neither on their own would have been sufficient to fully explain Darwin's observation. (*Top*: *Dynastes Hercules* (Dynastinae); *Middle*, *Left* to *Right*: *Golofa porteri* (Dynastinae); *Onthophagus rangifer* (Scarabaeinae); *Enema pan* (Dynastinae); *Bottom*: *Proagoderus tersidorsis* (Scarabaeinae); *Proagoderus lanista* (Scarabaeinae). The same approach can be used to understand the equally varied and wonderful forms of giant virus capsules (diameter > 0.2 μm), *Right*-hand panel. Fischer et al. used electron microscopy to discover what no amount of meta-barcoding data could tell them—what giant virus-like particles looked like (20). From just a few hundred grams of soil they discovered a higher diversity of morphotypes than all previously isolated giant viruses combined and named them appropriately as "Mimi-like" (*A*), "Supernova" (*B*), "Haircut" (*C*), "Turtle" (*D*), "Plumber" (*E*), "Christmas star" (*F*), "Flacon" (*G*), and "Gorgon" (*H*) as well as several with tail-like structures (*I*–*K*). The function of these forms is still mysterious but will only be solved considering both adaptation and mechanism, just as biologists have done in the beetle example. [Image credits: Left image is composite figure created by the authors from photographs courtesy of Doug Emlen. Right image shows viral capsid images reprinted from ref. 20, which is licensed under CC BY-NC-ND 4.0 (https://creativecommons.org/licenses/by-nc-nd/4.0/)].

New Ways of Seeing. The technological advances that have been developed to investigate biological mechanism at cellular and molecular level are starting to be used by biologists interested in adaptive function. We can now "watch and wonder" about the behaviors of individual cells and even molecules in the same way that we watch and wonder about the behavior of birds at a bird table.

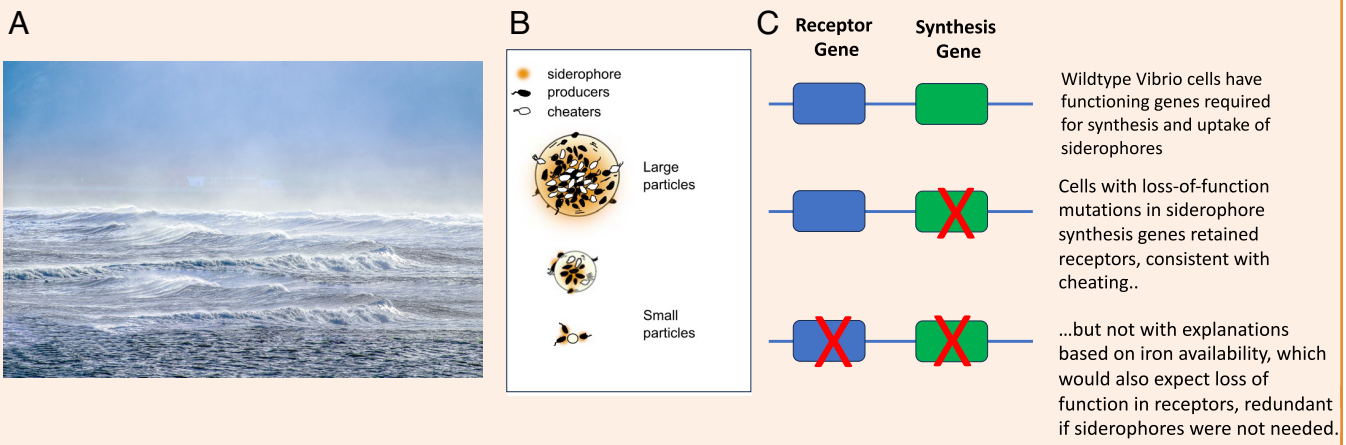
Interdisciplinary collaborations, which bring expertise in evolution together with expertise in technical innovation are expanding the possibilities for observing the invisible. One example comes from research on invisible behaviors performed by tiny organisms in a hidden world: mycorrhizae fungi living in the underground. Fluorescent nanoparticles (quantum dots) have allowed researchers to watch fungi move resources and trade with plants (29, 30). Technological advances in robotics and microscopy can perform dynamic observations of half a million nodes of mycelium network simultaneously (31). This has revealed the dynamic nature of fungal growth, showing how they move resources to areas where they are needed and where a better return on trade can be obtained.

This example nicely illustrates the value-added from combining an evolutionary approach to characterization of mechanism. A characterization of the mechanism of resource transportation cannot tell us about its function: Why mycorrhizae are transporting their resources to plants. It does not tell you about variation: why some mycorrhizae transport more resources than others. And it would not be able to make predictions about how farming practices could select for that transportation behavior to change (32). Only by understanding resource transport as an adaptation—how myccoryzae increase fitness through the formation of a trading-mutualism with plants—can do this.

The Matrix: Trading Off Realism with Control. In *The Matrix* film series, humans are deceived into thinking they are going about their daily lives in a 1990s city-scape, when, in fact, they are providing battery-power for evil robots. Biologists can adopt a similar strategy to the robots in this dystopian blockbuster and "trick" tiny organisms into thinking they are living in their natural environment when they are unwittingly part of a lab experiment.

There is nothing new about studying evolution in labs, and one advantage of bacteria and single-celled eukaryotes, such as the cellular slime molds (33, 34), is that they can be studied

Box 4.



Reading the genome to distinguish between adaptive hypotheses:

A. Bacterial cells are typically studied in a lab environment, where they no longer experience selection pressures that drove the evolution of the traits we are trying to understand. Can the behavioral ecology approach be used to understand the behavior of single cells living wild in the ocean? *B*: *Vibrio* bacteria live in the ocean and cluster together in particles of various sizes. Researchers observed that cells in larger particles were more likely to have mutations in genes responsible for the synthesis of iron-scavenging siderophores (55). Reprinted from ref. 54. *C*: They set about testing hypotheses about the functional explanations for this observation. They found patterns in the genome that were consistent with cells experiencing stronger selection to cheat in large particles, where siderophore-producing neighbors were more numerous. Nonproducers retained the receptors necessary to benefit from the plentiful supply of siderophore-bound iron, produced by cooperative neighbors. Retention of functioning receptor is not consistent with alternative explanations based on redundancy of siderophores in larger particles. [Image credit: Wikimedia Commons, licensed under CC 1.0. Anonymous, PxHere (pxhere.com/en/photo/1105848), CC0 via Wikimedia Commons (commons.wikimedia.org/w/index.php?curid=68416162)].

in the lab relatively easily (35). Micro-organisms can be grown in a test-tube, genetically modified with relative ease and, because they have relatively short generation times, they can be used in experiments which track the evolution of traits in real time (36–38). As well as contributing to our understanding of trait evolution in micro-organisms, this approach has been tremendously successful in contributing to our understanding of evolutionary processes more generally, such as parasite–host coevolution (39), resilience to stress (40), and cooperator-cheat dynamics (41).

Micro-organisms may provide tractable lab systems, but tractability often sits at odds with attempts to study adaptation: the more elegant and controlled the experiment, the more remote from nature (39). Studies of bacterial behaviors provide an example of this trade-off: it is possible to track the movement of individual cells in a fluidic device and compare patterns of behavior in much the same way as we study the behavior of animals. Such systems can be used to directly compare the ability of one strategy to out-compete another, the "gold-standard" experimental test of a hypothesis (42). But can we interpret the identification of a winning strategy in such an experiment as evidence that this strategy will have higher fitness in the natural environment?

Some species of bacteria are easier to "plug into the matrix" than others: *Staphylococcus aureus* is found naturally in human infections, relatively difficult to replicate in a lab. A pathogen of bean plants like *Pseudomonas syringii*, however, can be observed in its natural environment, simply by giving it a plant to grow on. An example of how we can make the most of opportunities to study bacteria in a more natural setting is provided by a study of spatial patterns of host–pathogen interactions by Saarenpää et al. (43). They developed a technique which maps microbe community composition, and host gene expression profiles over the surface of *Arabadopsis* leaves at the scale of a 100 μL grid. Previous techniques involved fluorescently labeling bacterial strains, or grinding up leaves—neither of which allow the complex spatial interactions between the microbiome and the host to be preserved. Plants were grown outside where they could be colonized by any passing microbe without being disturbed by a microbiologist.

The most useful approach will depend on the specific questions being asked. If we want to know the effect of iron-availability on the competitive dynamics between two strains of bacteria, a researcher might favor a more controlled in vitro system. If she wants to understand how selection drives variation in that trait more generally, she may need to consider systems that allow her to observe a response to selection in vivo. Similar trade-offs face researchers studying invisible life of all forms. For example, the behavior of malaria parasites can be studied in species that infect humans by growing them in vitro, in cell culture, or it can be studied in species that infect mice by growing them in vivo, in mice (44, 45). The best choice will depend on whether a researcher

needs to prioritize realism or relevance to human infection for their specific question of interest.

This is where *The Matrix* strategy can contribute. Lab systems can be designed that replicate the "real world" as closely as possible but still allow tiny behaviors to be observed. Problems only arise when we make our choices about the trade-off without full awareness of doing so. This error is more commonly committed through over-interpretation of results from controlled experiments, where expertise in evolutionary processes is lacking. Bacterial cells grown in liquid media, agar plates or fluidic devices are perhaps more like contestants in a game show such as *Squid Game* than denizens of *The Matrix*—they can be manipulated, pitted against one another in scenarios that require different skills, their every move can be watched, and strategies assessed. The observer will learn a lot about bacteria by watching them play but to what extent does behavior in an artificial game predict behavior in the real world (46)? Similar problems arise when interpreting the results of humans playing games in the behavioral economics literature (47).

Comparative Methods. If we want to understand why polar animals are white, we may hypothesize that whiteness is favored by selection for camouflage in habitats covered by snow. But how do we test this? We cannot perform an experiment (not practical) but we can test whether whiteness is more common in northern latitudes by applying the comparative method (48, 49). The comparative method takes advantage of patterns across species to test hypotheses about adaptation: Nature has done the experiment for us—some bears evolve in snow and others in forests—we just have to analyze the results. Statistical methods for such analyses are well developed, offering a diversity of ways in which to control for how species are phylogenetically related (e.g., how many times have bears independently evolved different colors) (48, 50). What is the scope for using this approach to understand adaptation in invisible things?

To carry out a successful comparative study, we need three things—a phylogeny (e.g., of bears), an estimate of the character trait we're interested in for species along the tips of that phylogeny (e.g., the color of bears) and third, an estimate of how our hypothesized explanatory variable is distributed across those tips (e.g., where bears live). There is no reason why we cannot fulfill these requirements for the traits of single-celled organisms or even cell-level behaviors within multicellular organisms. And there are already several good examples of studies that have provided insights into adaptive processes shaping behaviors of bacteria (51) and cells in multicellular systems (52, 53) that would have been impossible to generate any other way.

One example, uses a phylogenetic comparative analysis to understand a curious feature of DNA repair mechanisms across the tree of life (54). Double strand breaks, where both strands of DNA break together are potentially disastrous but can be repaired in the presence of an uncut copy of duplex DNA that can be used as a template. All domains of life rely on this mechanism. But some double-strand breaks occur in the absence of a template and, in this case, the only option is to trigger an emergency operation where the two strands are stuck back together again, in a process called nonhomologous end-joining. Sharda et al. showed that while this mechanism is ubiquitous in eukaryotes, it is only present in around 20% of prokaryote species (41). What is more, they found that the genes required to perform this repair are shared across the prokaryote phylogeny, even across domains, between archaea and bacteria.

Thinking like behavioral ecologists, Sharda et al. hypothesized that genome size might be selecting for the prevalence of nonhomologous end-joining. Bigger genomes are more likely to suffer from breakages and so, larger genomes create selection pressures to acquire these genes. They tested their hypothesis with a phylogenetic comparative analysis (the equivalent of mapping latitude to our hypothetical bear color analysis). The presence of genes associated with nonhomologous end-joining was correlated with genome size as they predicted.

No amount of detailed information about the mechanisms of DNA repair could have supplied this insight. By complementing their understanding of mechanism with a test of their hypothesis about adaptive function, they revealed something about (1) function—nonhomologous end-joining is an emergency measure and (2) variation—they were able to explain why some species have more of these genes than others and the means to (3) make a priori predictions about the likely frequency of genes for nonhomologous end-joining in a new species, using information about the size of its genome.

Footprints in the Genome. We may not be able to intuit potential selection pressures acting on life at the invisible scale but luckily, we have access to information that can indicate what those pressures may have been. The genome is the product of selection and we can make testable predictions about patterns of mutation, which can tell us something about how an organism has responded to selection or how selection operates on cells and genes within organisms.

This strategy has been adopted successfully to test hypotheses about molecular-level traits in bacteria living in sea water (55) and inside the lungs of human cystic fibrosis patients (56, 57) (Box 4). Bacterial cells, like all living things, cannot survive without iron, which they have to gather from their environment. They achieve this by secreting iron-scavenging molecules called siderophores, which they take up through specialized receptors in their cell membrane (58). In a study of *Vibrio* bacteria, living in the ocean, mutants defective in the ability to produce siderophores were observed (55). The frequency of these mutants varied, depending on whether they were sampled from a large or small groups of cells. Cordero et al. were able to test functional, adaptive explanations for this observation by analyzing patterns of mutations. The siderophore synthesis mutants had fully functioning receptors, which is consistent with a cheating "take-but-don't-give" approach to iron scavenging (9, 59).

The same principle was used to identify cooperator-cheat dynamics as the most likely explanation for loss of siderophore production in another species of bacteria, *Pseudomonas aeruginosa*, which infects the lungs of patients with cystic fibrosis (56, 57). These putative cheats were only found in the lungs of patients who were also infected with bacteria that produced the siderophore, pyoverdine. Furthermore, when all the pyoverdine producers were lost, then and only

then were mutations in the receptor genes observed. By asking the right questions, patterns of mutation can provide a means of distinguishing between competing hypotheses about selection pressures acting on organisms even if they live inside a living human being. In these examples, a mechanistic understanding of iron sequestration was complemented with an understanding of: (1) function— siderophore production increases fitness by sequestering iron for self and others; and (2) variation—why some individuals fail to express this trait, even in situations where it is expected to be beneficial. This information was then used to make (3) a priori predictions about the conditions where iron sequestration through siderophore release is likely to be found.

The revolution in genomic sequencing provides us with a treasure-trove of information about the selection pressures experienced over evolutionary time, in the environments in which an organism evolved. Evolutionary theory provides tools that we can use to generate testable hypotheses about adaptation in micro-organisms. For example, population genetic analyses predict that signatures in the genome ("footprints" of selection) can also be used to identify kin selection for cooperation (60, 61). If a trait has been favored by kin selection in nonclonal groups ($R < 1$), then cooperation will sometimes benefit nonrelatives that do not share the gene for cooperation. This reduces the kin selected benefit of cooperation, making beneficial mutations less likely to fix and deleterious mutations more likely to fix. Consequently, we predict that genes for cooperative traits favored by kin selection will show increased polymorphism and divergence, and higher rates of deleterious mutations. This method has been applied to traits which laboratory experiments had suggested were cooperative, such as siderophores and other public goods, in two species of bacteria: *P. aeruginosa* (62) and *Bacillus subtilis* (63). In both cases, the data supported the hypothesis that these traits had been favored by kin selection for cooperation. Automated methods for identifying genes for cooperative traits in bacteria mean that such studies now could be carried out relatively easily on a diversity of species (64).

The exciting thing about such population genetic methodologies is that they provide a window to watch and wonder about behaviors performed by micro-organisms living in natural populations, even when we do not have a detailed knowledge of the natural environment. We do not know how environmental conditions influence the selective benefits of traits, or the relative frequency with which different environments are encountered, or the average relatedness between interacting bacteria. These factors would be incredibly difficult to divine. But Mother Nature knows (65), and when she selects on alleles, she does so averaged across the different environments that are encountered. The population genetics pattern is a response to the evolutionary average natural environment and is measurable.

While our emphasis above has been the analysis of patterns in the genome to understand adaptation, we can also do the reverse and apply our understanding of adaptation to understand the genome. Genes are expected to respond to selection for improved ability to replicate and compete for space (on a chromosome) and/or resources (replication machinery). But even in cases where there are opportunities for genes to behave selfishly, selection at the level of the whole genome, sometimes described as "the parliament of the genes," can maintain order in the genome (66, 67). Evolutionary biology can help us generate testable hypotheses about patterns in the genome (68) and present excellent examples of the nuance and testability of the "behavioral ecology" method to operate in the genomic realm.

"The Problem with Evolution"

Despite the opportunities that exist to understand how natural selection operates on cells and genes, progress has been glacial. We suggest two possible reasons for this. First, behavioral ecologists have fully embraced technological advances in cell biology, genetics, and biophysics, but they have not embraced expanding their study systems of choice from visible to invisible life (3). Second, after decades of elucidating mechanistic details in ever-increasing detail, molecular biologists can lack the skills required to address "why questions." These skills require an understanding of the difference between Tinbergen's two categories of evolutionary questions and how evolutionary theory can be used to generate testable hypotheses. The generation and testing of hypotheses avoids post hoc "just-so" adaptive storytelling and an understanding of evolutionary theory is required to make sure that hypotheses are plausible (Box 1).

Systems biology is perhaps a reaction to growing recognition of the fact that mechanism on its own can only get you so far (69). A systems biologist attempts to place genes or proteins in context with "a system" as a whole by building models to simulate and predict how a biological system behaves under various conditions. For example, they might be interested in how cells respond to different stimuli, or how genetic networks influence development (70). Placing genes and proteins in a wider context is extremely valuable and has led to new insights and research directions (71). Problems can arise, however, when it is presented, implicitly or explicitly, as performing the same conceptual framework as Tinbergen's Four Questions. The motivation for the invention of systems biology may come from the same instincts that drove Tinbergen, but can lack his clarity of thought about the distinction between proximate and ultimate effects and how to generate formally justified a priori predictions.

This is a symptom of a wider problem, "*the problem with evolution,*" which is that, perhaps more than most other areas of biology, evolutionary biology suffers from the double curse of ignorance: many scientists do not know what they do not know about evolution (72). Like many of the best ideas, Darwin's theory of natural selection can be summarized on the back of an envelope Most students of biology can give you a perfectly acceptable description of how it works. This perhaps creates a false sense of confidence about how to take it into account in a study of, say, cancer or antibiotic resistance, which in turn can lead to speculative adaptive storytelling. Furthermore, it is still easy to find microbiology papers that suggest bacterial cells, or even their plasmids, are under selection to maximize parameters such as "population viability," which is not the predicted target of natural selection. The curious reader is directed to John Welch's insightful and entertaining discussion of why evolutionary biology, and behavioral ecology in particular, is

particularly vulnerable to the double-curse: "*Hypothesizing about adaptive rationales is easy to do badly, and difficult to do well*" (72).

The Solution

The behavioral ecology approach offers a solution to the problem of overwhelming bias in the life sciences toward mechanism; it does so by providing framework for developing and testing hypotheses about the adaptive function of traits (3). A particularly powerful outcome from the behavioral ecology approach and one that is not often available from mechanistic studies, is the scope for drawing general insights that apply across the natural world (*SI Appendix*, Table S1). To give a specific example, genetic relatedness has a clear and consistent influence on the evolution of cooperation across the entire tree of life, from bacteria and viruses to insects and birds (73). We see the same factors, such as the mechanism of group formation and kin discrimination, determine relatedness between social interactants time and time again across different taxa and across biological scales—genes, genomes, cells, individuals, and groups (74). This makes things pleasingly simpler for those studying the adaptive function of cooperation: Rather than requiring different explanations for different species, we have a single explanatory framework. Consequently, not only can we apply this approach to study the invisible world, but we can also generate conceptual links between the visible and invisible worlds. This unification is one of the crowning achievements of behavioral ecology.

Closing Remarks

This is an exciting time to be a biologist. After decades of advances in technology that allow us to watch individual molecules moving around in a cell in real time, or signals being transmitted through nerve fibers in the brain of a mouse; in an age where we have sequenced the genomes of thousands of organisms, the opportunity for expanding the range of questions we can answer is growing all the time. In the same way that Tinbergen moved the field of animal behavior from the lab and into the wild, there are now opportunities to apply his approach to test functional hypotheses about the behaviors of molecules and cells; both single-celled organisms but also cells that form the building-blocks of multicellular life. Failure to do this risks the same stagnation of progress in the life sciences as a whole that we would have witnessed in behavioral sciences if biologists never went into the field but restricted their enquiries to how animals behaved in lab experiments and zoos.

Data, Materials, and Software Availability. There are no data underlying this work.

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