Biol Philos
DOI 10.1007/s10539-016-9542-2

It's the song, not the singer: an exploration of holobiosis and evolutionary theory

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Received: 25 July 2016 / Accepted: 7 October 2016

Abstract That holobionts (microbial communities and their animal or plant hosts) are units of selection squares poorly with the observation that microbes are often recruited (horizontally acquired) from the environment, not passed down vertically from parent to offspring, as required for collective reproduction. The taxonomic makeup of a holobiont's microbial community may vary over its lifetime and differ from that of conspecifics. In contrast, biochemical functions of the microbiota and contributions to host biology are more conserved, with taxonomically variable but functionally similar microbes recurring across generations and hosts. To save what is of interest in holobiont thinking, we propose casting metabolic and developmental interaction patterns, rather than the taxa responsible for them, as units of selection. Such units need not directly reproduce or form parent-offspring lineages: their prior existence has created the conditions under which taxa with the genes necessary to carry out their steps have evolved in large numbers. These taxa or genes will reconstruct the original interaction patterns when favorable conditions occur. Interaction patterns will vary (for instance by the alteration or addition of intermediates) in ways that affect the likelihood of and circumstances under which such reconstruction occurs. Thus, they vary in fitness, and evolution by natural selection will occur at this level. It is on the persistence, reconstruction, and spread of such interaction patterns that students of holobiosis should concentrate, we suggest. This model also addresses other multi-species collectively beneficial interactions, such as biofilms or biogeochemical cycles maintaining all life.

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Published online: 13 October 2016

Keywords Holobiont · Hologenome · Biofilm · Unit of selection · Natural selection

Introduction

Animals and plants together with all the microbes in and on them are now often called "holobionts", and the ensembles of their genomes "hologenomes" (Zilber-Rosenberg and Rosenberg 2008; Bordenstein and Theis 2015; Theis et al. 2016). Three claims might be taken as implicit in this language: (1) that many of the microbial and macrobial taxa associated in such multi-species assemblages are metabolically (or developmentally or structurally) interdependent, (2) that such interdependence has co-evolved as a consequence of their association, and (3) that holobionts can be considered units of selection. A similar set of ideas informs research and philosophizing about biofilms, structured microbial communities embedded in an extracellular matrix, not necessarily associated with an animal or plant (Clarke 2016).

It is our view that both holobionts and biofilms are so disunified as classes that no *general* resolution of such claims can reasonably be expected. The focus of the now quite heated debate around the widespread use of "holobiont" and "hologenome" is most often on claim (3) above. What primarily undermines this claim, that holobionts (or biofilms) as collectives might be units of selection, is the failure of the lineages they comprise to *reproduce as collectives*—to exhibit coordinated vertical inheritance (Douglas and Werren 2015; Moran and Sloan 2015; Clarke 2016). That is, for many of the entities currently called holobionts (or biofilms) there is no mechanism guaranteeing that the several component or partner organisms of a parent entity are the parents of the component or partner organisms of an offspring entity. Instead, new microbial partners are recruited (horizontally acquired) from environmental reservoirs in each generation. Sometimes the new partners are taxonomically identical to their predecessors and co-evolution may well have occurred, each of a pair of lineages harboring genes selected to affect its interaction with the other. But often, new partners are similar only in performing the same role in the collective: they belong to the same functional "guild" as their predecessors, but not the same taxa (Burke et al. 2011). And pairwise, species-on-species coevolution involving a macrobe and hundreds or thousands of microbial lineages independently seems unlikely, if not impossible (Douglas and Werren 2015). There is no obvious mechanism by which the latter could evolve collectively (but see below as to how they might appear to do so).

So, if the idea that microbial consortia are units of selection is to be borne out, it might be better to focus on evolutionary processes at a *functional* level, not the taxonomic level of partner organisms and their lineages. Put metaphorically, what matters is the song, not the singer. The song, to flesh out this metaphor, is the pattern of interactions (metabolic, structural, or developmental) between partner lineages (the singers). Here, we argue that interaction patterns as entities do in fact vary, pass on traits to later instances of themselves, and differ in propensity to spread and persist; they thus act as units of selection. Making the case that interaction patterns fulfill the criteria associated with units of selection does require a relatively

substantial rethinking of various evolutionarily relevant concepts, especially that of "reproduction." The revision we propose may seem radical, but we suggest that it can save what is interesting in holobiont thinking and perhaps usefully broaden evolutionary theory.

Multilineage systems and evolution

One of the fruits of amazingly rapid progress in DNA sequencing and bioinformatic analysis is a new appreciation of the vital indirect and direct roles of organisms we cannot see, microbes, in the lives of those we can (macrobes) and in the biosphere as a whole. Although we have known for many decades that major biogeochemical cycles in the ocean or on land are driven by microbes and that microbes affect animal, human, and plant health directly in many ways, not all negative, the 21st century sciences of metagenomics and microbiomics have created new interest in the public and drawn the attention of philosophers and philosophically inclined scientists. Much research proceeds with the unexceptionable goal of uncovering the many important microbe–macrobe interactions neglected in the necessary effort to reduce the toll taken by frank pathogens (Charbonneau et al. 2016; Sonnenburg and Bäckhed 2016). But the new methodology has allowed a vast expansion in research scope and along with this has come a new evolutionary microbial ontology (for review, see Doolittle 2013; Booth et al. 2016).

Holobionts

Particularly appealing to many are claims that microbial communities together with associated host macrobes are evolutionary individuals, indeed possibly units of selection. In one of the founding documents in this field, Zilber-Rosenberg and Rosenberg write…

> In the hologenome theory of evolution, we suggest that the holobiont… (the host and its symbiotic microbiota) with its hologenome, acting in consortium, should be considered a unit of selection in evolution, and that relatively rapid variation in the diverse microbial symbionts can have an important role in the adaptation and evolution of the holobiont (Zilber-Rosenberg and Rosenberg 2008, 723–724).

Although there is a significant theoretical literature on what it is that is selected even in single lineages, microbiologists and holobiont enthusiasts alike have not often attended to the nuances of such discussions, and are often imprecise in their use of philosophically contested language. What does seem clear is that enthusiasm for holobionts and biofilms largely derives from the notion that the lineages that make them up are some sort of holistic and cohesive unit, and that they have undergone something like what Queller would call an egalitarian transition. Such transitions are…

> egalitarian in the sense that both partners reproduce (although not necessarily equally). The advantage of such alliances is the bringing together of two disparate units with distinct capabilities, a combination of function, which in the new entity becomes a division of labor. The greatest barrier to such alliances may be the potential for the two parties to exploit each other, but such conflicts can be limited if there is sufficient mutual interdependence (Queller 1997, 186).

However, interdependence comes in degrees, and embedded in the phrase "unit of selection" is something like the assumption that holobionts have gone the further integrative step that characterizes what Maynard Smith and Szathmáry called "major transitions in evolution", namely that "entities capable of independent replication before the transition can replicate only as part of a larger whole afterwards" (1995, 6). It is such an implication, expressed for instance by Bordenstein and Theis, that has caused most debate, and motivates our discussion here:

> Hologenomic evolution is most easily understood by equating a gene in the nuclear genome to a microbe in the microbiome… Evolution for both genes and symbionts is fundamentally a change in population frequency over successive generations, i.e., the fraction of holobionts carrying that particular nuclear allele or microbe (Bordenstein and Theis 2015, 3).

Holobiosis enthusiasts envision an inevitable transformation of all evolutionary biology once holobiont thinking is taken fully on board. Consider, for example, the claims of an interdisciplinary team comprising a noted developmental biologist, a historian of biology, and a philosopher of medicine, writing in a recent review with the existentially evocative subtitle "We have never been individuals":

> …recognizing the "holobiont" – the multicellular eukaryote plus its colonies of persistent symbionts – as a critically important unit of anatomy, development, physiology, immunology, and evolution opens up new investigative avenues and conceptually challenges the ways in which the biological subdisciplines have heretofore characterized living entities (Gilbert et al. 2012, 325).

Bordenstein, one of the holobiont's strongest supporters, is hopeful for the future and transformative potential of such concepts.

> Today, it is convention that mitochondria represent anciently acquired bacteria that have a fully integrated partnership with the animal genome. The challenge ahead for biology is to resolve whether fractions of the environmentally acquired, but host-associated beneficial microbiome can be understood in a similar way. The hologenome concept emphasizes that all of these entities comprise the genetic repository of the host organism. It allows for a holistic view of the evolutionary process. Evolutionary geneticists and microbial ecologists currently see this issue from diverse vantage points, and thus merging and resolving these views is an essential and potentially transformative frontier for biology (Bordenstein 2013, 261).

Perhaps unsurprisingly, there has been pushback from more conservative philosophers and microbiologists over what would justify claims that holobionts ought rightly to be called units of selection. One overriding issue is the heterogeneous nature of the class of objects addressed. There is a well-populated spectrum of degrees of physical, metabolic, and reproductive integration, at one end of which there are undoubtable units of selection, however defined, and at the other just assemblages of lineages. Another is how often established co-evolutionary theory will satisfactorily accommodate the available facts. That theory, as we conceive it, entails the separate fixation in two or more species of traits enhancing fitness because they promote trans-specific interaction (positive or negative: parasites do co-evolve with hosts).

Among philosophers, Booth (2014), for instance, has argued that there must be collective *reproductive lineages,* definable at some level, on which selection might impinge. Booth contrasts the individuality problem of holobionts with that which lateral gene transfer (LGT) poses for the "Tree of Life". Although LGT compromises talk of *genomic lineages* for prokaryotes, at least it is possible that there are reproductive lineages of prokaryotic *cells* (Doolittle and Brunet 2016; Booth et al. 2016). But for many of the loose macrobe–microbe relationships considered to be holobionts by the authors cited above, there are no coherent collective reproductive lineages. Thus…

> … there are determinate cell-level lineages in prokaryotes that exhibit a branching, tree-like structure, while there are not in most holobionts. Holobionts, that is, exhibit determinate lineages among their parts (the analog of gene lineages in prokaryotes), but no determinate lineages among the collectives (no analog of cell lineages in prokaryotes) (Booth 2014, 668).

Another, related, concern is that the microbiomes of hosts are often treated as causal actors affecting host biology as a cohesive unit, despite the fact that microbiomes are very often composed of diverse microbes participating in diverse interactions with the host and with each other (something that holobiont enthusiasts readily acknowledge). Douglas and Werren (2015), for instance, note that…

> A host plus its microbiome is more effectively viewed as an ecological community of organisms that encompasses a broad range of interactions (parasitic to mutualistic), patterns of transmission (horizontal to vertical), and levels of fidelity among partners. The hologenome requires high partner fidelity if it is to evolve as a unit. However, even when this is achieved by particular host-microbe pairs, it is unlikely to hold for the entire host microbiome, and therefore the community is unlikely to evolve as a hologenome (Douglas and Werren 2015, 1).

Moran and Sloan (2015), after a well-argued critique, conclude that…

> The central claim of the hologenome concept, that a host and its microbiome together form the primary unit of selection, is sometimes true, and sometimes false; its validity will depend on the particular case. …, we suggest that the varied and often inconsistent interpretations of the hologenome concept have

> been a source of more confusion than clarity in understanding the evolution of host–microbe interactions. Advances will come from empirical studies that start with rigorous assumptions and a clear framework for detecting coevolution and teasing apart levels of selection (Moran and Sloan 2015, 8,10).

O'Malley (forthcoming) envisions that the emphasis on collective community function will fade as more individual microbe–macrobe interactions come under mechanistic scrutiny; we suspect she is right.

However, backers of more inclusive holobiont/hologenome thinking have very recently responded to critics such as Douglas and Werren and Moran and Sloan, reasserting in a 15 author statement of principle the value of holobiont thinking (Theis et al. 2016). They argue that the idea of holobionts as units of selection was never the sole focus of their theoretical explorations, that Moran and Sloan have mounted a "straw man argument" (Theis et al. 2016, 3), and that "holobionts and hologenomes" are "not restricted to one special process but constitute a wider vocabulary and framework for host biology in light of the microbiome" (Theis et al. 2016, 1). This framework allows that "hologenomic variation may arise not only by mutation and recombination in the host and microbiome but also by acquisition of new microbial strains from the environment, a change in microbial abundance, and horizontal transfer among microbes" (Theis et al. 2016, 2). Such variability notwithstanding, these authors insist that "one can look at holobionts and hologenomes as incontrovertible realities of nature" (Theis et al. 2016, 2). But it is arguably the case that abandoning any requirement for holobionts to be units of selection and/or to reproduce collectively, is, for all intents and purposes, throwing out the baby with the bathwater. We revisit this issue at the end of this section.

Biofilms

Biofilms can comprise cells of a single or several species: those of the latter sort concern us here (Røder et al. 2016). As communities they are physically held together by an extracellular matrix, with different species or strains occupying different internal or external territories, doing different but often complementary biochemistries, communicating by chemical signals, and exchanging genes at an enhanced rate by LGT, also responsive to such signals. Biofilms can be but are not necessarily the microbial parts of holobionts: their "hosts" can just as easily be rocks or the bottoms of boats as they can macrobes.

In biofilm philosophizing there is also a move to see collectives as real and evolutionarily cohesive even though collective (vertical) reproduction can be supplemented (sometimes 100 %) by (horizontal) recruitment. Ereshefsky and his colleagues have been especially active in promoting multi-species biofilms as organism-like evolutionary individuals, endorsing a relaxation of some traditional requirements for collective reproduction. Ereshefsky and Pedroso ask…

> What are we to make of such multispecies biofilms? If numerous multispecies biofilms are good candidates for evolutionary individuals, then standard reproductive requirements on individuality should be reconsidered. We do not

> deny that such criteria highlight important factors that contribute to the individuality of single-species eukaryote organisms. However, the case of multispecies biofilms (and other multispecies consortia) indicates that evolutionary individuality is achieved through other means than often-cited reproductive processes. We suggest that the existence of such multispecies individuals shows the need for a pluralistic and open-ended account of evolutionary individuality (Ereshefsky and Pedroso 2015, 10126).

Clarke has, very recently in these pages, challenged Ereshefsky and Pedroso's suggestion from several angles. Although she also mistakenly takes our position (elaborated below and in Doolittle 2013) to be theirs, her critique has merit and mirrors those of Douglas and Werren (2015) and Moran and Sloan (2015) with respect to holobionts. Clarke finds that

> Thanks to the aggregative nature of biofilm formation, and to the retention of reproductive independence by cells, there will rarely be enough genetic heritability across biofilm generations to support a response to selection… on balance, there is little utility in treating wild biofilms as if they can function as evolutionary individuals. In other words [she doubts] that wild biofilms generally evolve by group selection, where whole biofilms are taken as groups (Clarke 2016, 208).

We doubt this too.

Evolution

What we see happening, then, is an attempt to preserve the interest in and momentum of holobiont and biofilm research by relinquishing the necessity of collective reproduction (and thus the status as "major transitions" in evolution in Maynard Smith's and Szathmary's sense). In their place we are offered assertions about how often it is that macrobes and microbes occur together (as "incontrovertible realities") in nature—a surprise only to nonmicrobiologists—or attempts to recast them as interactors at the collective cellular level (Ereshefsky and Pedroso 2015; Lloyd forthcoming). We think both are mistakes insofar as they render claims about holobiosis (and in particular assertions about the reality of hologenomes) of limited ontological interest, or untrue as a general rule. Co-evolution needs no alternative theory, defining holobionts as the microbiota of macrobiota smacks of "eukaryocentrism" (Booth and Doolittle 2015), many of the most interesting cases (the bugs in our own guts for instance) are quite far from the collective reproduction needed for a proper (major) transition, and some macrobe–macrobe symbiosies (fig wasps and their hosts, e.g.) have crossed that line more convincingly.

We suggest that there is a different way to think about the evolution of holobiotic systems, one that importantly diverges from the overtly organismal lineage-based considerations often taken to be of vital importance in contemporary discussions. Our starting point, elaborated in the next section, is one that has been acknowledged by holobiont proponents: the microbial taxa that partner with any given host macrobe or with each other can be highly variable taxonomically—not the same

strains, the same species, or even, sometimes, the same phyla. Many holobionts nevertheless maintain relative functional stability by recruitment of microbial taxa, physiologically or biochemically equivalent but not necessarily of the same genetic lineages, from the environment. This kind of stability has undoubted evolutionary significance. Figure 1 summarizes the model we entertain.

It's the song, not the singers In an early comparative metagenomic study by Turnbaugh and co-workers, such functional stability is described in ecological language. Characterizing microbial community samples from 154 people, the authors conclude. …

> The hypothesis that there is a core human gut microbiome, definable by a set of abundant microbial organismal lineages that we all share, may be incorrect… Instead, it appears that a core gut microbiome exists *at the level of metabolic functions*. This conservation suggests a high degree of redundancy in the gut microbiome and supports an ecological view of each individual as an 'island' inhabited by unique collections of microbial phylotypes: as in actual islands, different species assemblages converge on shared core functions provided by distinctive components (Turnbaugh et al. 2007, 483, emphasis ours).

This is now the common finding: in sites like the guts of different healthy humans, where similar ecology is expected, similar "functional genes" (encoding metabolic activities involved in the exploitation or production of local resources) are found. The microbial taxa bearing such genes however often prove to vary, especially when the taxonomic analysis is fine grained enough to differentiate species and strains (The Human Microbiome Project Consortium 2012; Boon et al. 2014). That means that there is considerable metabolic redundancy, genes for the same function being distributed across many taxa, so that whatever combination of activities support a "eubiotic" (healthily functioning) gut can be assembled in many ways, recruiting from environmental diversity. The title of a recent paper on ruminant microbiomes (Taxis et al. 2015)—"The players may change but the game remains"—expresses exactly the same sentiment we articulate as "It's the song, not the singer(s)" in the ideas presented here (see also Doolittle 2013).

Functional metabolic processes uncoupled from taxonomy (at least at a fine scale) are found not only in microbiomes inside the intestines of macrobes (holobionts) but also in microbial communities in decaying dead whales at the bottom of the sea, on our teeth, comprising plant mycorrhizae, and at a host of other natural and human-made sites (Wade 2013; Laranjo et al. 2014; Tringe et al. 2005). For instance, from a comparison of the microbiota on the surfaces of different *Ulva australis* (a seaweed) individuals, Burke et al. conclude that…

> Despite the high phylogenetic variability in microbial species composition on different *U. australis* (only 15 % similarity between samples), similarity in functional composition was high (70 %) … This observation of similarity in habitat (niche) use with respect to functional genes, but not species, together with the relative ease with which bacteria share genetic material, suggests that

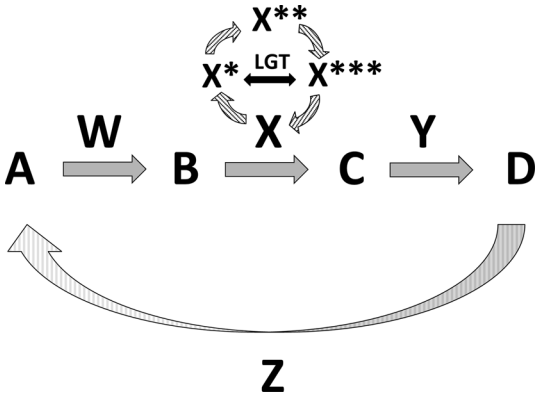

Fig. 1 A general representation of the relationship between songs and singers. *A–D* are intermediates in a metabolic pathway or biogeochemical cycle, stages in a developmental process, different components of a macromolecular assembly or any combination thereof that can be the collective product (the "song") of the interactions between multiple microbial and/or macrobial taxa (the "singers"). *W–Z* are those singers, and their interactions can be either linear (without *Z*) or cyclical (with *Z*). *X**, *X*** and *X**** are other taxa of the same "guild" as *X*, not necessarily closely related to it phylogenetically but capable of making the same contribution to the collective activity. Often their capacity reflects LGT of relevant genes. *X* can and often will be replaced by others of its guild and similar substitutions can occur with taxa *W*, *Y* or *Z*. Since each of the singers knows its own part, the song may be temporarily interrupted. The singers will take it up again, such recurrence being the equivalent of replication. Alterations of the interaction pattern (such as new intermediates or subsidiary cycles or further complexifications of developmental processes) are equivalent to mutations, can and do occur, and will be passed on through replication by recurrence. Thus the song itself can evolve by natural selection, and its existence (as a "constructed niche") will stimulate the evolution of guilds, often comprising thousands of microbial species

> the key level at which to address the assembly and structure of bacterial communities may not be species (by means of rRNA taxonomy), but rather the more functional level of genes (2011, 14288).

These authors suggest that an appropriately ecological microbial classification would recognize functional "guilds," phylogenetically heterogeneous groups defined only by their possession of one or more biochemical capacities (Burke et al. 2011). Similarly, Thomas et al. report from a broad survey of sponge microbiota that…

> Our findings support a model of independent assembly and evolution in symbiont communities across the entire host phylum, with convergent forces resulting in analogous community organization and interactions (2016, 1).

They also note that…

> Sponges can maintain highly diverse, yet specific symbiont communities, despite the constant influx of seawater microorganisms resulting from their filter-feeding activities. These symbioses are known to be at least partially underpinned by metabolic exchange between symbiont and host, including nitrogen cycling, CO2 fixation, secondary metabolite production, and uptake and conversion of dissolved organic matter. In this respect, sponge symbionts perform analogous functions to the symbionts found in mammalian guts and plants (2015, 2).

Some functionally similar microbial communities in similar environments exhibit such similarity because they are directly (vertically) inherited (and therefore also show taxonomic identity). For instance termite microbiomes, including all relevant taxa, are passed from mother to daughter by "proctodeal trophallaxis" (anus to mouth) (Noda et al. 2007). In other cases, species-to-species co-evolution is ensured by horizontal recruitment of the same microbes, (sameness being determined via 16 rRNA analysis). A good example would be the much discussed bobtail squid, in which the (microbiologically sterile) newly hatched squid recruits *Vibrio fischeri* from the surrounding ocean to populate its developing light organ. The colonizing bacterial genome shows evidence of having evolved to grow in its host, and the squid has many genes dedicated to forming a light organ (Rader and Nyholm 2012). This is to be sure a mutualism, and it may be that squid-friendly vibrios are concentrated near squids, but the evolutionary fates of the lineages of *individual* squids and their personal vibrios are not linked: there is no collective reproduction, only species-on-species co-evolution. So there are no proper evolutionary individuals or units of selection, as far as individual holobionts are concerned.

Many cases are like that of *U. australis* and the sponges cited above: presumably microbiomes are functionally similar but taxonomically different in both cases because only microbes capable of thriving on algal surfaces or in sponges are recruited and/or retained, but these comprise guilds with many phylogenetically diverse taxa. Since both *Ulva* and sponges have ecologically distinct single cell or larval dispersal stages, adults are unlikely to have inherited all their microbes from their parents. For something like the human gut microbiome, where the maternally inherited microbiome (in vaginal birth) is replaced early in childhood (Koenig et al. 2011), the fact that adult microbiomes bear even a loose similarity to each other must be due to similar patterns of recruitment and/or maintenance based on gene function, not on taxonomy *per se*. Sometimes, but not necessarily, such function will entail "eubiosis"—a microbiome promoting host health. However, as Moran and Sloan note…

> … observing host-specific microbial community composition or greater community similarity among more closely related hosts does not imply that symbionts have coevolved with hosts, let alone that they have evolved for the benefit of the host (Moran and Sloan 2015, 1).

Sometimes, microbes will just be in the gut because it is a good place for them to grow. And "dysbiotic" microbiota can also become established, to dramatic effect as in recurrent *Clostridium difficile* infection (Rao and Safdar 2016) and more subtly in a host of human disorders (Clemente et al. 2012; Cho and Blaser 2012).

Interaction patterns as units of selection

So, how might we imagine that it is the song (the collective metabolic and other interaction patterns that support putative holobionts) and not the singers (the sometimes variable and horizontally acquired organismal lineages that carry them out) that is primarily of evolutionary interest in holobionts and biofilms? What sort

of generally Darwinian theory might accommodate the fact that, during healthy adult life, we humans maintain a relatively constant suite of "eubiotic" microbial metabolisms and interactions even when the taxa underwriting this condition vary between us and over time? Is it a problem that such identity over time as there is does not necessarily persist over host reproductive cycles, but is instead reconstructed anew each generation with different taxonomic building blocks?

Approaches to a theory might already be seen in the literatures on LGT and on homology. Omelchenko et al. (2003) offer a list of "mosaic operons," linked genes determining proteins involved in a definable biochemical pathway or macromolecular complex, in which one or more genes has been derived by "orthologous replacement" (LGT from a different lineage). In several such cases (the ribosome unarguably, but likely also F_0F_1 ATPases, respiratory complexes, and tryptophan biosynthesis), it is reasonable to assume that a pathway or complex was present and functioning without interruption, even as one of its components was replaced. Thus the pathway can have been maintained in two species derived from a common ancestor employing that pathway—that is, maintained as homologous—by gene recruitment.

Formally similar cases, in which homologous anatomical structures (likely present in ancestors) are produced by non-homologous developmental processes (cell–cell interactions) have been addressed by Sommer (2008) and by Wagner (2007, 2014). Wagner argues that "it is the historical continuity of gene regulatory networks rather than the expression of individual homologous genes that underlies the homology of morphological characters" (Wagner 2007, 473). There are, in Wagner's perspective, lineages of networks that exhibit something like parent-offspring relationships not underwritten by genes or cells. Regulatory networks of this type are in a sense patterns only, functionally characterized relationships that can be said to bear at least minimal relations of ancestry and descent. It is not clear where such entities would belong in any overtly materialist biological hierarchy (cells, organisms, species, etc.), whether they are outcomes of major transitions, or even whether any explanatory goal would be served by answering such questions. Nevertheless they are arguably recognizable songs, performed by a panoply of diverse genealogically embedded singers over the course of evolutionary history.

We propose a similar perspective on the multilineage metabolic networks and other interaction patterns that are often instantiated by holobionts and other microbial consortia (Fig. 1). These are persistent functionally characterizable patterns that, despite being uncoupled from specific taxa, coherently exhibit relations of identity, and which change over evolutionary time. Our model requires that instances of metabolic networks and other interactions can be identified as belonging to the same type at different times and places, even given substantial divergence in the material constituents that provide the basis for the relationships. Materialistic considerations, having to do with which cell lineages are involved in distinct manifestations of metabolic relationships, are explicitly backgrounded. Songs can be performed by choruses composed of different singers, in different venues at different times, perhaps employing distinctive arrangements or unusual instrumentation in each instance, but all are recognizably versions of the same song.

Our proposal is that metabolic relationships and interaction patterns ought also to be considered along these lines.

Once that step has been taken, our model proposes that such metabolic songs are units of selection. We largely agree with Godfrey-Smith when he writes that

> To ask whether something is a unit of selection – either in general or in a particular case – we should ask whether those entities vary, pass on traits in reproduction, and differ in reproductive success. The same test is applied in all cases, including genes, organisms, groups, species, artifacts and ideas. For some of these objects it is hard to work out what reproduction involves, but that is what to look for… These questions arise especially for collectives, where there can be evolutionary processes at many levels at once" (Godfrey-Smith 2015b, 74).

Godfrey-Smith himself notes that most holobionts, conceived as clusters of lineages that may be physiologically or developmentally integrated, are not themselves units of selection (Godfrey-Smith 2013). This is primarily because of horizontal acquisition of symbionts, which encourages a view according to which holobionts are not evolutionary units in their own right, but rather complex communities of interacting lineages that evolve independently. We agree with this perspective, but it is one that emphasizes the patterns of transmission of cellular lineages making up holobionts and not the abstract functional relationships that those lineages engage in. The abstract metabolic or developmental songs we are interested in here might be considered as being closer to Godfrey-Smith's "ideas" than they are to genes or organisms.

At this point, some readers might understandably be thinking that it is indeed "hard to work out what reproduction involves" when what is purportedly being reproduced are abstract, functionally characterized metabolic pathways or other interaction patterns. We outline our approach to this issue in the next section.

Recruitment, recurrence, reconstruction

In a recent review on the history of microbial ontology, one of us (Doolittle 2013) argued for considering interactions patterns more specifically as a kind of replicator. [It is this view that (Clarke 2016) mistakes for endorsement of microbial taxa themselves as the units of selection]. In describing "community interaction patterns as replicators", Doolittle wrote…

> Suppose a functional microbiome (essential to the survival of its host) comprising organisms of types A, B and C is more-or-less stable because A feeds B, B feeds C and C feeds A, and collectively they benefit their host. Loss of B would create an opportunity for any taxon B* that could feed from the products of A, and those B* recruits that could also feed C would be selected at the level of host survival. But B and B* need not be (though they might be) of the same "species" or even phylum. (They *would* be of what Burke et al. 2011 call the same "guild", importing a term from classical ecology). Such

> heritable interaction patterns would be subject to mutations (alterations in what A feeds B or potential variants B* for instance) and exhibit variable fitnesses (mediated through their interactions with hosts) (Doolittle 2013, 371).

When microbiomes are directly passed (vertically inherited) from parent macrobe to offspring macrobe, or when biofilms replicate by multispecies bits breaking off, then such a process entails "material overlap" (Griesemer 2000), involves some "bottlenecking" (Bonner 1974), and might meet common criteria of *reproduction* (Godfrey-Smith 2009). But often what happens is not that. Functionally similar (but taxonomically non-identical) microbes are recruited from the environment each generation. Godfrey-Smith would describe the recruitment of similar microbiomes across macrobial generations as instances of *recurrence* through *reconstruction*, not *reproduction*, and would thus consider holobionts that are formed in this way to lack the parent-offspring *lineage* relationship required of units of selection…

> Recurrence of structure is a general feature of living systems, seen both in things that reproduce and things that do not. Reproduction and reconstruction are two different causal bases for recurrence. Structures such as hearts and ribosomes recur because they are reconstructed, from generation to generation or on some other temporal scale. Reproduction generates parent-offspring lineages between instances of recurring structures, whereas reconstruction does not generate such lineages… Organisms are metabolic units. In principle, such units might arise from either reproduction or reconstruction. We usually think of organisms as things that reproduce, but it is possible to put pressure on that idea (Godfrey-Smith 2015a, 10122).

When the focus is on the lineages of organisms that make up holobionts, then it is difficult if not impossible to track parent offspring lineages, and one risks stretching the concept of reproduction beyond its breaking point (Booth 2014). However, when the focus is on the reconstruction of metabolic and other interaction patterns across generations, and not on the lineages of organisms that make them up, one might be tempted to consider the possibility that such reconstruction is a kind of "formal reproduction," and that there is indeed "a chain of material *influence* linking parent and offspring, without the parent supplying a crucial piece of matter that initiates the new individual" (Godfrey-Smith 2009, 81). Substituting interactions patterns for organisms does "put pressure" on the reproduction/reconstruction boundary.

There are some real and not so dissimilar biological situations involving recurrence via reconstruction in which no one would doubt that evolution by natural selection (ENS) is at play. If the true genome of a retrovirus is RNA in a virion, then that vanishes as its sequence gets copied into the DNA of a host genome-embedded provirus, only to be reconstructed much further down the road (maybe even host generations later) when the provirus is transcribed into RNA for packaging in virions. Replication of single-stranded DNA and RNA viruses involves reciprocal templating: no "crucial piece of matter" winds up in the offspring molecule. And prions (also cited as potential formal reproducers by Godfrey-Smith) only use recruitment: no new material is made.

A better analogy might be cultural entities, indeed songs and singers. Multi-part songs recur each time singers sing them, but lineages of singers knowing only their own parts (perhaps learning them from parents) might never have heard the whole piece through, until brought together to sing it again. Different songs or different vocal arrangements of the same songs will compete for popularity, and comprise lineages, but that does not mean that any particular singing of one arrangement might be said to the parent of a singing of it in the future. Singing sessions are continual but need not be continuous. Similarly, the nitrogen cycle (without which all life would grind to a halt) requires the services of nitrogen fixing bacteria (often in root nodules but also heterocystous cyanobacteria free in the ocean), nitrifying bacteria, denitrifying bacteria, plants (assimilating nitrates), and decomposers, each a guild of uncountably many species and strains, often owing their contributing genes to LGT. If by some thermodynamic or quantum miracle all nitrogen fixation stopped for the next 10 min, who would doubt that it would start up again right away, or question that, once started, it was in some sense "the same" cycle?

Indeed, there is a long and multifaceted history of argumentation over whether or not ENS can proceed through recurrence alone, often conducted as part of the debate over niche construction. One thread of this history begins with Patrick Bateson's review of *The Selfish Gene* in which he offered, as a *reductio ad absurdum* of Dawkins' portrayal of genes as the only real and eternal biological replicators, the notion that nests (reconstructed recurrences in Godfrey-Smith's framework) are also replicators, making copies of themselves through the agency of birds (Bateson 1978, 2006).

Sterelny et al. (1996) asserted, contrariwise, that this view and indeed many others that recognize that "it is arbitrary to single out genes as the units of selection" are basically correct. They write of nests…

> There is a flow of information linking nest generations through the builders. Nests and burrows are adapted for the growth of burrow builders and nest-makers. Those interactors carry the information through which the nest is replicated. Bateson was right: sometimes, perhaps always, nests meet Dawkins' definition of a replicator, and they meet ours too (Sterelny et al. 1996, 400).

On the model proposed in this paper, interaction patterns established by varying lineages of microorganisms can be conceived similarly. Such patterns are in a sense adapted for the benefit and spread of the microorganisms that participate in their functions. In turn, those interactors (microorganism collectives) carry information enabling the reconstruction of future similar interaction patterns. Interaction patterns are akin to formal replicators in the sense that they are reconstructed via material *influence* as opposed to material *overlap*, but unlike traditional biological replicators in the sense that they are not subcellular parts such as genes, transposable elements, or prions. We do not in general favor the replicator/interactor model (Hull 1980) as the best or only way to understand selection, but it is worth noting that it can be coherently employed in this context, and in a way that is substantially different from other proposals. Lloyd (forthcoming), for example, conceives holobionts as interactors that promote the differential success of the lineages of cells

that make them up. We offer an alternative in which holobionts are seen as interactors that promote the differential success of the interaction patterns they instantiate. The replicators in our model are abstract functional relationships, not cell lineages.

Another helpful contrast is with the work of De Monte and Rainey, who have very recently presented a model in which recurrence plays the role of reproduction in what they consider ENS, applying it to the origin of multicellularity and collectives such as the human microbiome. They seek to replace Lewontin's (1970) heritable variation in fitness formulation with "[1] identity: a criterion for identifying collectives… [2] recurrence: a relationship between collectives at time t and time t" > t such that at both times the collectives are characterized by the same identity criterion … [and 3] genealogy: the possibility of identifying the precursor(s) of a recurrence, based on the sharing of particle lineages among collectives across successive recurrences" (De Monte and Rainey 2014, 242–243). With respect to the human microbiome, they write…

> A typical stance would be to ask to what extent do these communities manifest heritable variance in fitness. But, as pointed out above, this requires the existence of measurable parent-offspring relationships… Our relaxation of Lewontin's conditions provides a way forward: the issue is whether there exists a genealogical relationship between recurrences. This can be addressed at different temporal and spatial scales. All that is necessary is a means of sampling and a means of genotyping. Both are possible. Indeed the data likely currently exist (2014, 247).

We think these authors have gone too far in not requiring some specified causal source or connection between recurring instances of "the same" collective, but not far enough insofar as they have not abandoned collectives of material entities as the units of selection. Our model sees recurrences of a particular metabolic pathway or interaction pattern (each song) as ultimately being caused by previous instances of the same pathway or pattern through the agency of the many microbes (singers) that the pattern has called into existence—something like niche construction (Odling-Smee et al. 1996), of wide scope and long duration (see Fig. 1). ENS of interaction patterns is in a sense uncoupled from that of the holobionts or microbial communities that carry them. Lineages are not defined by parent-offspring relationships between organisms or collectives of organisms, nor by traceable histories of individual instances in which interaction patterns are implemented. And to think of a pattern itself as parent to any particular implementation of it is a category error, just as it would be to conflate songs (abstract and potentially "immortal") with specific performances of them (spatiotemporally restricted and concrete). Nevertheless, interaction patterns differ in their propensities to spread (multiple instantiation) or persist as these are determined by the evolutionary dynamics of lineages and genes that carry out their steps. A "fitter" interaction pattern (more favorable to its host or more easily acquired by recruitment) may arise quickly, for instance by interposition of a new or more efficiently accomplished step consequent upon acquisition of a new microbial lineage or gene, but its spread depends on the proliferation of that gene or lineage. Locally this might be very

rapid, as recent studies of microbial microecological adaptation indicate (Datta et al. 2016), but global spread may be protracted. The evolution of patterns is no less complexly caused and unpredictable than that of organisms or species.

How it all started and is maintained Morowitz et al. seek to reconcile metabolism-first and replication-first scenarios for the origin of life by distinguishing greediness—"in which existing states of order compete to increase their own instantiation at the expense of others (2008, 7)"—and individuality, as it is usually cashed out for genes, organisms or self-replicating RNAs. In "Selfish metabolism" they write …

> Such a metabolism would also naturally entrain the order of the structure built on it, in that whenever a molecule or structure arises at a higher level and enhances core metabolism, it is recruited via a feedback loop or loops to remain part of the developing complexity of the evolving structure. In extant life, this feedback dependency can be seen at the biochemical, cellular and organismic levels. Darwinian competition among organisms emerges with the advent of individuality, from a deeper molecular competitive exclusion that statistically selects the inputs from which all life must be assembled. The appeal to metabolism can be seen as much when a cofactor develops to enhance reactions that incorporate ammonia into amino acids, as when a Lake Turkana crocodile develops a jaw structure enabling it to catch and eat Nile perch (Morowitz et al. 2008, 8).

We, in what may be an alternative formulation, have sought to retain individuality (broadly construed) and assign it to interaction patterns, while recognizing that few can be discretely delimited and that often there will be multiple intraspecies and interspecies connections between patterns and networks (perhaps akin to epistasis of genes). We imagine that metabolic pathways (the simplest interaction patterns to conceptualize) whose steps are catalyzed by different microbial taxa (species or strains) might have arisen sequentially and collectively, each added partner deriving some energetic or nutritional advantage by using another partner's leaked intermediate or excreted waste product as a substrate (Lenton and Watson 2011). Alternatively, pathways might arise within a single organismal lineage by either of the two popular scenarios [retrograde or patchwork evolution (Jensen 1976; Rison and Thornton 2002)] and then become distributed across several descendant lineages through streamlining selection or neutral processes (Fullmer et al. 2015; Gray et al. 2011).

Many biological and environmental constraints will of course be determining factors. Common metabolites will play a directive role: Schmidt et al. observe …

> … a strong bias among enzymes in the use of metabolites. These frequently used metabolites play an important role because new pathways seem to evolve around these compounds with a preference (2003, 340).

Once in place, a pathway provides niches for strains that catalyze its steps to better advantage, to compete and supplement or sometimes supplant each other, establishing guilds. Often, it is at the level of genes, laterally transferred between

existing or newly joining (thanks to the transfer) guild members that such competition occurs. When provision of services to some microbial host is at issue, it is not necessary that the macrobe and all microbial members of the guild co-evolve on a pairwise basis. Co-evolution of a host with a single microbial partner can establish host genes serving that interaction: other microbes (or genes if LGT plays a role) then evolve with the host serving as an "environment" that need not further co-evolve.

Thus pathways or more generally interaction patterns in holobionts are themselves constructed niches, created by the earliest performers of their individual steps but then setting up conditions in which very many additional taxa capable of performing the same steps (or improved versions thereof) are continually selected for (as in Fig. 1). Because there's a song there are singers: because there are singers, there's a song. It is even more tempting to think this way when there is no macrobial host, as in some biofilms or indeed, perhaps, the biosphere as a whole (Louca et al. 2016). Shared metabolisms are part of the fabric of Nature (Falkowski et al. 2008): very recent metagenomic sequencing surveys have uncovered communities made up of multiple bacterial or archaeal cellular lineages whose genomes lack one or another gene necessary for independent growth (Brown et al. 2015). Indeed, communities in which the steps in biochemical pathways necessary for the survival of many members are divided up among them can be modeled as, under some circumstances, evolutionarily inevitable (Fullmer et al. 2015; Mas et al. 2016).

In sum

Bordenstein asks, "whether fractions of the environmentally acquired, but host-associated beneficial microbiome can be understood in a similar way" to mitochondria within eukaryotic cells (2013, 260). We hold that as long as "environmentally acquired" means that microbial partners of the parent holobiont are not reliably the parents of the microbial partners of its offspring, the answer is no. When environmental acquisition is always of the same microbial species or strain and *either* the host or the microbe has evolved mechanisms to recognize its partner, one can be taken as the environment of the other. When *both* are true, co-evolution theory can be applied. This does not require us to think in a way similar to any theory of eukaryogenesis (Booth and Doolittle 2015). The vast majority of a macrobe's microbes are likely to be in or on it because this is to their individual advantage. But this is not to say that hosts have not evolved to take account of their possession of a microbiota, or that microbiota have not evolved in response to their hosts. Nor is it to say that there are not complex and mutually beneficial shared metabolisms and developmental interactions within microbial communities and microbe–macrobe partnerships. The question is how we are to understand their evolution within any formal theory of ENS.

Our model requires that ENS can occur in populations of differentially reconstructed or perhaps differentially persisting entities (Doolittle inpress). The populations we envision comprise varying interaction patterns, differing in the nature and complexity of their activities and components. Such individuals are

differently fit depending on the ease with which microbes and the genes of microbes can evolve to carry out their steps, because it is through their agency that interaction patterns are reconstructed. Although there are lineage (parent-offspring) relationships between different interaction patterns, there are not parent-offspring relationships between instances of the same pattern. An interaction need not be continuously in operation for the evolved microbiota to resume its performance after an interruption: they are singers that already know the song. Neither a composition-based "major transitions" nor a "levels of selection" approach will accommodate this model: songs are perfomed by—but do not comprise—their singers.

Thinking this way reverses the normal causal chain and, we think, gets at the heart of what has always been of interest in holobiont research, although many details will undoubtedly require further elaboration. Rather than seeing shared metabolisms as the products of some sort of group selection operating on individual lineages—or, in any rare mitochondria-like cases, on some hologenome—to create multilineage interactions, we imagine that such interactions already exist. Lineages evolve to carry out their steps because in each case it is selectively advantageous to individuals (or their genes) within those lineages to do so. There is no need to envision the independent evolution, by some onerous collective mechanism, of similar patterns in thousands of individual holobiont species.

We are left with one question: "Where did such pre-existing interaction patterns come from?". Morowitz et al. (2008) allude to an answer, and the question of how multistep interaction patterns arise is not more difficult whether one imagines them to emerge within single lineages, to always have been shared by multiple lineages, or to have originated within single lineages and then been parceled out. Nor is it a question unique to our theory, which is one about maintenance rather than origins. It is the maintenance by diverse and inconstant microbial taxa of the quasi-stable metabolic and developmental interactions that sustain not only supposed holobionts and biofilms but all life on this planet that we have aimed to address.

Acknowledgments We thank the Natural Sciences and Engineering Research Council of Canada (Grant GLDSU 447989) for support and Maureen O'Malley, Carlos Mariscal, Tyler Brunet, Letitia Meynell, and Andrew Fenton for comments on an earlier version.

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