

Animal-Microbe Interactions in the Context of Diapause

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Abstract. Dormancy and diapause are key adaptations in many organisms, enabling survival of temporarily or seasonally unsuitable environmental conditions. In this review, we examine how our understanding of programmed developmental and metabolic arrest during diapause intersects with the increasing body of knowledge about animal co-development and co-evolution with microorganisms. Host-microbe interactions are increasingly understood to affect a number of metabolic, physiological, developmental, and behavioral traits and to mediate adaptations to various environments. Therefore, it is timely to consider how microbial factors might affect the expression and evolution of diapause in a changing world. We examine how a range of host-microbe interactions, from pathogenic to mutualistic, may have an impact on diapause phenotypes. Conversely, we examine how the discontinuities that diapause introduces into animal host generations can affect the ecology of microbial communities and the evolution of host-microbe interactions. We discuss these issues as they relate to physiology, evolution of development, local adaptation, disease ecology, and environmental change. Finally, we outline research questions that bridge the historically distinct fields of seasonal ecology and host-microbe interactions.

Introduction

Many organisms have evolved diapause, a genetically programmed developmental arrest, as an adaptation enabling survival of temporarily adverse environmental conditions. Diapause is characterized by reduced metabolic rates, arrest of morphogenesis and reproductive functions, and resistance to

environmental stresses. The capacity to express diapause is widespread across animal taxa such as fishes, nematodes, and arthropods and allows survival through conditions such as winter cold or summer drought (Tauber *et al.*, 1986; Hand *et al.*, 2016). Obligatory diapause occurs at a specific, genetically determined stage and is therefore controlled by endogenous factors only. Facultative diapause is regulated by environmental signals that are predictive of unfavorable conditions, such as a decrease in photoperiod and/or temperature before winter. Other environmental stimuli, such as moisture or resource availability and quality, may also modulate diapause responses (Danks, 1987). Intra- and interspecific interactions, such as the presence of predators or strong competition for resources, can also affect diapause expression (Kroon *et al.*, 2008; Tougeron *et al.*, 2018). The expression of diapause is species specific; different species express diapause at different developmental stages and modulate their development and physiology during diapause to different extents. Diapause influences community ecology and maintenance of biodiversity because diapausing stages can allow for long-distance dispersal, especially among aquatic invertebrates (Panov *et al.*, 2004); diapausing stages can form biotic reservoirs of genetic and species diversity in “seed banks” of cysts or resting eggs (Hairston, 1996).

Although diapause and other types of dormancy are widely recognized as important evolutionary strategies for animals coping with seasonal or fluctuating environments, understanding how these metabolic and developmental changes may affect or be affected by microbes has received almost no attention. Host-microbe interactions are increasingly recognized as universal forces in animal evolution (McFall-Ngai *et al.*, 2013; Douglas, 2014b). For example, conflict with pathogens has favored the development of mechanisms for the generation of genetic diversity (*i.e.*, sex) and of metabolically costly lines of defense, including behavioral adaptations, physiological barriers, and innate and adaptive immune systems. Cooperation with beneficial symbionts has enabled animals to occupy new

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Abbreviation: FOXO, forkhead transcription factor.

niches by adopting ready-made adaptations, such as the production of vitamins, fermentation products, or bioluminescence. And tolerance of omnipresent bacteria has shaped animal physiology by “tuning” and modulating myriad physiological processes. Microbes, like animals, are affected by and evolve in response to changes in their environment; when their environment consists of an animal host that itself responds to environmental conditions, envisioning the trajectory of microbial communities and populations becomes more complicated (Costello *et al.*, 2012; Carey and Duddleston, 2014). Based on these considerations, there is reason to believe that microbes could mediate physiological processes during the diapause of their animal hosts (Table 1) and, conversely, that diapause of animal hosts should have a strong impact on the host microbiome.

There are a number of aspects of diapause biology that intersect with studies of host-microbe interactions. Diapause involves dramatic metabolic remodeling, and microbes form a key component of host metabolism, both because of metabolic processes that microbes carry out and because of their system-wide pleiotropic effects on immunity and function. More broadly, diapause is an example of developmental plasticity, in which microbes can play important roles (Gilbert *et al.*, 2015). During diapause maintenance periods, when development is arrested until endogenous or exogenous signals allow it to resume, animals are exposed to predators, parasites, and pathogens. During diapause, some vital functions have to be maintained, at least at a low level, to ensure survival. These include nutrition, immunity, toxin evacuation, cold and heat resistance, or prevention of water loss; thus, effective management of energetic resources to maintain these processes during diapause is essential (Hahn and Denlinger, 2011). Microorganisms can affect most of these functions in non-diapausing organisms; it is therefore reasonable to consider whether they also affect them in the context of diapause. Finally, microbes both modify and respond to the environment, making them potentially an integral component of the signals about environmental conditions

to which dormant animals respond. From the perspective of the host-microbe system as a whole, diapause introduces a potentially important challenge for the synchrony and maintenance of interactions between hosts and the microbiota.

This review explores direct and indirect links between microorganisms and animal diapause. We focus on arthropods but also consider informative examples from other taxa. Because diapause is important for coping with seasonality, these examples are frequently closely tied to seasonal phenomena generally. In recognition that diapause strategies exist along a gradient, with different features of interest in diverse taxa, we likewise draw examples from a range of dormancy phenotypes, from hibernation and torpor to reproductive diapause (arrest of reproductive functions), to prolonged, genetically programmed “deep” diapause. We examine how seasonal environmental variation could affect the microbiome either directly or indirectly through changes in host physiology during dormancy. Conversely, we speculate on the roles that microorganisms might play in regulating seasonal strategies, within host lifetimes or over evolutionary time. We are also interested in the broad question of what it means for animal-microbe interactions when the continuity of an animal’s lifespan is interrupted and then resumes a significant length of time later. Both diapause strategies and host-microbiome interactions have been extensively reviewed from diverse perspectives (Tauber *et al.*, 1986; Danks, 1987; Dethlefsen *et al.*, 2007; Denlinger, 2008; Kostic *et al.*, 2013; McFall-Ngai *et al.*, 2013; Douglas, 2014a). Our aim is to highlight underappreciated areas of intersection of these disparate fields in hopes of stimulating more interdisciplinary work on this topic.

Do Microbes Influence Diapause Entry and Exit?

There is currently little direct evidence that gut or intracellular microbes could be cues *per se* for diapause initiation or termination. However, there is evidence for cross-talk between

Table 1

Nonexhaustive list of examples of potential types of host-microbe interaction effects on diapause phenotypes

Research area	Mechanisms of interest	Relevant literature
Microbial effects on host diapause preparation, entry and exit	• Cross-talk between microbes or immune pathways and metabolic and developmental pathways involved in diapause • Roles of microbes as environmental cues	Shin <i>et al.</i> , 2011; Zheng <i>et al.</i> , 2017; Lee <i>et al.</i> , 2019 Kaul <i>et al.</i> , 2014; Khanna <i>et al.</i> , 2016; Samuel <i>et al.</i> , 2016
Microbial functions during diapause	• Microbial effects on seasonal strategies; cold and heat tolerance • Nutrient regulation and recycling during diapause • Antibiotic production during diapause • Other stress tolerance mechanisms?	Neelakanta <i>et al.</i> , 2010; Corbin <i>et al.</i> , 2017 Kashima <i>et al.</i> , 2006; Liu <i>et al.</i> , 2016; Wiebler <i>et al.</i> , 2018 Kaltenpoth <i>et al.</i> , 2005; Koehler <i>et al.</i> , 2013 Unknown
Host-pathogen interactions during diapause	• Diapause as pathogen avoidance strategy • Diapausing hosts as reservoirs for pathogens • Immune regulation during diapause	Palominos <i>et al.</i> , 2017 Ullman and McLean, 1988; Nasci <i>et al.</i> , 2001; Nelms <i>et al.</i> , 2013 Denlinger, 2008; Kubrak <i>et al.</i> , 2014

microbes and numerous signaling mechanisms involved in animal developmental pathways, including some that are involved in diapause transitions. For example, the insulin-like peptide signaling pathway and activation of forkhead transcription factor (FOXO) are high-level regulators of diapause entry in insects, nematodes, and fish (Sim and Denlinger, 2013; Woll and Podrabsky, 2017). FOXO activation and insulin-like peptide activity change in response to bacteria (either those in the native microbiome or specific infections) in invertebrate hosts (Dionne *et al.*, 2006; Shin *et al.*, 2011; Zheng *et al.*, 2017). Similarly, the production of a specific form of the universal insect life-history regulator juvenile hormone (Flatt *et al.*, 2005) was recently shown to be stimulated by the presence of a specific bacterial symbiont in bean bugs (Lee *et al.*, 2019). Further links can be found in studies of circadian biology, which is relevant because circadian rhythms are implicated in diapause initiation in response to changes in photoperiod (Hand *et al.*, 2016). Host-microbe interactions are affected by the circadian rhythms of the host, and microbes can reciprocally manipulate host rhythms in several ways (Wier *et al.*, 2010; Heath-Heckman, 2016; Lendrum *et al.*, 2017). In general, the common biochemistry underlying immune, metabolic, endocrine, and developmental pathways merits consideration when examining the possibility of microbial influences on diapause (Becker *et al.*, 2010; Broderick, 2015; Neuman *et al.*, 2015; Fischbach and Segre, 2016).

The scant handful of direct observations of microbes influencing diapause comes from studies of pathogens and food bacteria. Pathogens can affect diapause induction, either as a pathogenesis strategy or as a side effect of host fitness reduction. Females of the crustacean *Daphnia magna* infected by the microsporidian *Hamiltosporidium tvaerminnensis* produced fewer diapausing eggs than uninfected females (Sheikh-Jabbari *et al.*, 2014). Strains of *Trichogramma* parasitoid wasps infected with the reproduction-manipulating bacterium *Wolbachia* experienced disturbed expression of clock genes, reduced diapause, and lowered overwintering fitness than uninfected strains (Rahimi-Kaldehy *et al.*, 2017a, b). Infection can also end dormancy, as observed in bats, where the fungal infection that causes white nose syndrome prematurely ends the animals' winter torpor (Warnecke *et al.*, 2012). Several more examples of bacteria influencing diapause entry and exit come from *Caenorhabditis elegans* nematodes, which feed on bacteria and can facultatively enter a stress-resistant diapause-like form called dauer. Dauer formation differs in response to different types of bacteria, as assessed using either diverse natural bacterial isolates or panels of bacterial mutants (Khanna *et al.*, 2016; Samuel *et al.*, 2016). Some, but not all, bacterial fatty acids appear to be a signal for exiting dauer (Kaul *et al.*, 2014). Furthermore, when fed on pathogenic bacteria, *C. elegans* produced offspring that formed dauers; and dauers formed even after several generations that were not exposed to pathogens, suggesting transgenerational transmission of a dauer formation signal (Palominos *et al.*, 2017). Under laboratory

conditions, *C. elegans* interactions with microbes are typically limited to the *E. coli* used as food, whereas *C. elegans* isolated from natural environments has more diverse interactions with a distinctive species-rich microbiome (Dirksen *et al.*, 2016); therefore, there may be underappreciated microbial effects on dauer dynamics under natural conditions.

Microbes can also have indirect effects on diapause through global effects on host metabolism and life history. Exit from photoperiod-induced reproductive diapause in olive flies (*Bactrocera oleae*) appeared to be affected by the combination of dietary composition and presence of bacteria in the fly diet (Koveos and Tzanakakis, 1993). In aphids, it can be argued that microbiota can indirectly regulate diapause by altering the production of specific aphid morphs. Aphids overwinter as diapausing eggs that are produced after sexual reproduction. In the pea aphid *Acyrtosiphon pisum*, the facultative bacterium *Spiroplasma* induces a male-killing phenotype, thus reducing the proportion of males (Simon *et al.*, 2011) and potentially reducing production of diapausing eggs in the population.

Microbial communities within hosts can shift seasonally (see *Changes in Microbiome Composition During Diapause*, below); and hosts may be sensitive to such changes, which could represent indirect cues initiating or terminating diapause. Studies of seasonal changes in microbial associations have, to date, not examined whether these changes are cues for diapause initiation or consequences of factors associated with diapause (e.g., abiotic environmental changes or diapause-associated changes in host physiology). Furthermore, microbes in the external environment, which also shift in response to environmental changes (Waldrop and Firestone, 2006; Lennon and Jones, 2011; Gilbert *et al.*, 2012), either could modify the environment in a way that is conducive to diapause entry or exit or could serve as cues. These possibilities are worth examining because microbes could be useful reliable signals about the environment (Douglas, 2014b); if particular microbes are surviving and thriving, after all, it is an unmistakable sign that the environment is favorable for them to do so. There is precedent for such a phenomenon in the observation of microbes as environmental signals triggering other types of metabolic and life-history transitions. For example, metabolic products produced by gut bacteria trigger satiety and cessation of feeding in the mammalian host; production of these products is directly related to the amount of food consumed (Breton *et al.*, 2016). In a more dramatic example of a phenotypic transition cued by microbes, tubeworms and some other marine invertebrates use signals from surface-associated bacteria to settle on those surfaces and metamorphose from free-floating larvae into their adult sessile form (Shikuma *et al.*, 2014). If microbes were found to trigger diapause entry or exit, we speculate that one advantage of evolving to sense microbes as a cue is because microbial activity might serve as an integrated signal of several different environmental factors (Gilbert *et al.*, 2015). For example, if a microbe requires a particular combination of nutrients, salinity, and temperature in its

environment to survive and produce a particular metabolite, and if this combination of parameters happens to coincide with conditions favorable for an animal surviving after exiting dormancy, then evolving to sense the microbial metabolite during diapause might offer a greater chance of survival than evolving to respond to just one of these environmental factors. Conversely, it is also possible that some bacteria could evolve to trigger diapause transitions for selfish ends, for example, breaking dormancy in hosts in order to parasitize them when they become metabolically active. These possibilities are especially worth exploring in the context of diapause exit, because the endogenous or environmental processes responsible for diapause termination are generally less understood than those for diapause entry in most species (Hand *et al.*, 2016).

Changes in Microbiome Composition During Diapause

When an animal enters diapause, the first question that arises with respect to the microbiome is whether its microbial associates survive (Fig. 1). If they do, then the question arises as to whether the microbiome also becomes dormant or remains metabolically active, followed by the question of whether continued bacterial metabolic activity is to the benefit or the detriment of the host. Another possibility is that the bacteria transition to a free-living existence independent of the host. All of these outcomes could occur differently for different members of a diverse microbial community; in all of these scenarios, the parallel question arises: what mechanisms have hosts evolved to control or cope with diapause-associated changes in microbiota?

A number of studies of wild animals have examined seasonal changes in their microbiome (Ludvigsen *et al.*, 2015;

Maurice *et al.*, 2015), often connecting these changes to seasonal shifts in diet, temperature, or infection status. Comparatively fewer studies have specifically investigated the effect of host dormancy on the microbial community. The effect of changing seasons on the microbial community will depend on the extent to which a host habitat insulates the microbial community from seasonal changes in the environment. In invertebrates and other ectothermic organisms, microbes will be subject to the same temperature changes as their hosts, so host diapause-related changes may be less relevant than changes induced by winter itself, as reviewed in, for example, Corbin *et al.* (2017). In endotherms, the thermal conditions experienced by bacteria are likely to change less dramatically during winter in metabolically active animals than they do in hibernating animals. Aside from clear examples such as stability *versus* instability of host temperature, the extent to which diapause changes a host's capacity to act as a physical refuge for bacteria is not well understood. A related question is how the physiological changes in the host change the conditions under which different bacterial species compete.

In several systems, diapause may be a stage when diverse microbial communities are winnowed down to a core subset. Medina *et al.* (2018) defined “non-transient” microbiota as those that persisted in the midgut of *Nezara viridula* (southern green stinkbug, a soybean pest) during diapause. Some of these bacteria had the capacity to deactivate soybean chemical defenses, suggesting that the insects are able to maintain potentially valuable bacterial partners in their gut during diapause. Marine copepod microbiota are highly variable from individual to individual but undergo some predictable changes when the copepods stop feeding and enter diapause (Datta *et al.*, 2018). Specifically, bacterial taxa associated with

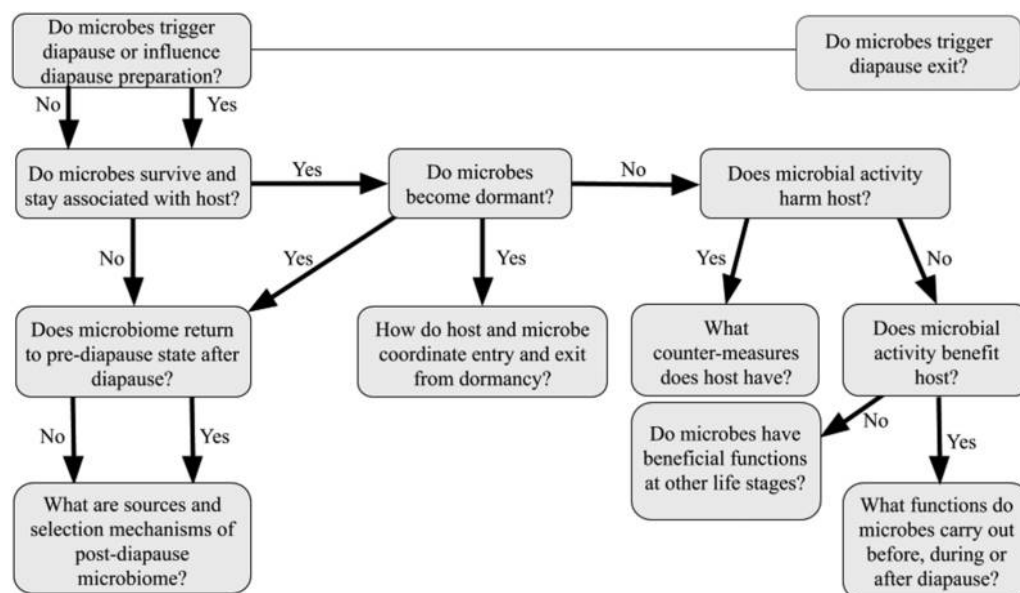


Figure 1. Questions concerning the dynamics of interactions between diapausing animals and their microbiota.

phytoplankton decline in relative abundance; this could be either because the copepods are no longer consuming these bacteria along with the phytoplankton or because these bacteria residing in the copepod guts no longer have an influx of phytoplankton to feed on. In the European forest cockchafer *Melolontha hippocastani*, which undergoes a long diapausing stage as an adult, a subset of eight taxonomic classes of bacteria persisted in the diapausing gut (Arias-Cordero *et al.*, 2012). Thus, despite the different food sources (roots vs. leaves) of adults and larvae, and despite the long period of starvation separating them, continuity of a subset of the microbial community was maintained. However, in all of these examples, it is unknown whether the bacteria were themselves dormant or metabolically active during host diapause and what (if any) regulatory mechanisms in the host controlled these bacterial populations.

Of particular interest is the composition of the microbiota in organisms in which the diapausing stage is an embryo. Is a diapausing embryo colonized with microbes that it carries through diapause to the post-diapause stage, or does it first acquire its microbiome after exiting diapause? Transovarial transmission of intracellular symbionts is generally considered to be the most reliable method for ensuring microbial persistence across host generations, but even this mechanism appears to be altered by diapause (Peccoud *et al.*, 2014). In pea aphids, the intracellular defensive symbiont *Regiella insecticola* was perfectly vertically transmitted from parents to offspring through parthenogenetic, non-diapausing eggs but was occasionally lost from sexually produced diapausing eggs (Moran and Dunbar, 2006). Similarly, *Wolbachia* appears to be occasionally, unpredictably lost from the parasitoid wasp *Nasonia vitripennis* after prolonged diapause maintained by refrigeration (Perrot-Minnot *et al.*, 1996). In contrast, *Wolbachia* actually increased in titer during overwintering in the cricket *Gryllus veletis* (Ferguson *et al.*, 2018), suggesting that this bacterium could be differently affected by cold and/or host dormancy, depending on the host species or life stage. Few studies have examined the extracellular microbiome associated with embryonic diapausing stages. When we investigated the bacteria inside and outside the resting egg cases (ephippia) of *Daphnia magna*, we found that there were indeed bacteria encapsulated inside the egg case (Mushegian *et al.*, 2018). However, it was bacteria on the outer surface, rather than the inner surface, that were most important to *Daphnia* fitness after hatching, despite the great interindividual variation in microbial community composition between resting eggs. This suggested that environmental acquisition after diapause rather than maternal provisioning is the primary mechanism by which *Daphnia* obtains beneficial microbiota.

Microbiome Functions During Diapause Preparation and Maintenance

Cooperation with microorganisms is a widespread strategy that allows animals to “outsource” certain functions to micro-

bial partners. For example, many phytophagous insects depend on microorganisms to provide essential nutrients that are not readily available in the insect’s diet. How such cooperative partnerships are stabilized and maintained is a vast field of study. Consideration of diapause introduces novel specific questions to this area of inquiry. A particularly relevant question is whether microorganisms carry out any such useful physiological functions during host dormancy. On the one hand, by definition, host metabolic requirements are reduced during dormancy, so many microbial partners likely become unnecessary during this time. For example, in the sycamore aphid *Drepanosiphum platanoidis*, which depends on the endosymbiont *Buchnera* for amino acid provisioning, reproductive diapause during the summer appears to be accompanied by suppressed symbiont activity (Douglas, 2000). On the other hand, outsourcing functions to microbes during dormancy may be part of the strategy that enables hosts to shut down these functions. Numerous transcriptomic studies have examined gene expression in diapausing *versus* non-diapausing arthropods (Ragland and Keep, 2017); extending these studies to examine microbial gene expression might provide clues to microbial function in diapausing hosts.

Most of the available evidence of physiologically important microbial functions during diapause falls into the categories of carrying out starvation metabolism and producing defensive antibiotics. The shield bug *Parastrachia japonensis* enters reproductive diapause and survives without feeding for several months, during which recycling of nitrogenous compounds, such as uric acid, to produce amino acids is essential. During host diapause, uricase activity appears to be carried out by *Erwinia*-like gut bacteria; removal of these bacteria leads to reduction of amino acids in the hemolymph and rapid death of the host (Kashima *et al.*, 2006). Nitrogenous waste recycling was similarly carried out by bacteria in the guts of hibernating frogs (Wiebler *et al.*, 2018). In preparation for reproductive diapause, the metabolism of female cabbage beetles shifts from protein to lipid synthesis; in concert with this, the composition of the microbiome also shifts slightly, leading the authors to speculate that the microbial community may be involved in metabolic regulation in a manner similar to regulation of adiposity by microbiota in mammals (Liu *et al.*, 2016). In hibernating mammals, the composition of the microbial community during diapause shifts to become dominated by bacterial taxa that feed on host epithelial mucins (Carey *et al.*, 2013; Dill-McFarland *et al.*, 2014), similar to what occurs when there is insufficient fiber in the diet (Sonnenburg *et al.*, 2005). These bacteria appear to produce baseline levels of short-chain fatty acids, even while the host is not eating (Carey and Duddleston, 2014; Stevenson *et al.*, 2014). Furthermore, the microbial community as a whole appears to be involved in mediating hibernation-related metabolic plasticity, since transplanting the summer and winter microbial communities of bears into mice resulted in different patterns of weight gain and adiposity (Sommer *et al.*, 2016). However, in hibernating

mammals, microbial metabolic activity during hibernation is associated with occasional inter-bout arousal, when body temperature temporarily increases, whereas, presumably, no such thermal regulation occurs in invertebrates such as the shield bug. Thus, it would be of interest to understand which microbially mediated metabolic activities can and cannot continue in the absence of regulation by the host.

In addition to microbial modulation of primary metabolism during diapause, there is currently one example of bacteria augmenting secondary metabolism during host dormancy, namely *via* antibiotic production. Females of the European beewolf (*Philanthus triangulum*) cultivate *Streptomyces* bacteria in antennal glands, which they use to infect brood cells prior to oviposition. The developing larva, which has a nine-month diapause stage within a cocoon, survives at significantly higher rates in the presence of the symbiotic bacteria, probably because of antibiotic production and protection from fungal and bacterial infections that are common in soil-nesting insects (Kaltenpoth *et al.*, 2005). Antibiotic production by the bacteria appears to coincide temporally with the production of the cocoon (Koehler *et al.*, 2013), suggesting the existence of some regulatory mechanism that controls bacterial antibiotic production at the diapausing beewolf's most vulnerable stage. Protection of eggs by antibiotic-producing bacteria is known in other non-dormant species (Lindquist, 2002), but the factors that allow or favor the persistence of this kind of defense mechanism over a long period of host dormancy are currently unexplored.

Pathogenicity and Virulence During Diapause

During diapause, arthropods face increased exposure to parasites and pathogens, including bacteria, fungi, and viruses, especially when diapause occurs at immature, immobile stages. These forms cannot behaviorally escape parasites or potential infection zones and are weakened by metabolic depression (Tauber *et al.*, 1986). In most insect species, immunity is maintained or even enhanced during diapause (Nakamura *et al.*, 2011; Kubrak *et al.*, 2014). For example, in *Drosophila melanogaster*, some genes involved in innate immunity are upregulated during reproductive diapause, independently of acute infection (Kubrak *et al.*, 2014). It is unknown to what extent this immune upregulation depends on previous or ongoing contact with microbes, given the evidence for microbial regulation and the priming of baseline innate immunity (Cirimotich *et al.*, 2011; Sansone *et al.*, 2015). Defense compounds specifically produced by diapausing arthropods are likely to be of interest for pharmacological prospecting and human health (Denlinger, 2008), and studying the mechanisms used by hosts to suppress pathogen replication during diapause could provide clues for strategies to manage pathogen transmission under non-diapause conditions. The cricket *Gryllus veletis* has decreased survival during overwintering as a result of fungal infection but also appears to benefit from reductions of pop-

ulations of potential pathogens in the gut during overwintering (e.g., *Pseudomonas* spp. and *Serratia marcescens*) (Ferguson *et al.*, 2018). In some taxa, host susceptibility to pathogens during diapause can be used for biological control. For example, entomopathogenic nematodes are used to control codling moth (*Cydia pomonella*) in orchards by specifically targeting sensitive diapausing larvae (Lacey and Chauvin, 1999). Conversely, diapause could also be an escape strategy that helps organisms avoid contact with infectious agents (Palominos *et al.*, 2017).

Parasites and pathogens often survive detrimental periods together with their hosts; and diapausing hosts can serve as reservoirs for pathogens, thus facilitating pathogen outbreak in spring (Denlinger, 2008). For example, the winter form of the pear psylla (*Psylla pyricola*) represents an overwintering reservoir for *Phytoplasma* bacterial parasites of plant phloem during its reproductive diapause (Ullman and McLean, 1988). Similarly, viruses such as West Nile virus can overwinter in diapausing adult *Culex* mosquitoes and can be transmitted into eggs (Nasci *et al.*, 2001; Nelms *et al.*, 2013). At the population scale, the spread of diseases and pathogens among individuals is often an indirect consequence of the diapause state. For example, parasites and pathogens with density-dependent transmission modes can spread rapidly within populations that aggregate for overwintering, such as ladybugs (Webberley and Hurst, 2002). An indirect consequence of adult diapause is that it increases the amount of overlap between generations of adults, which allows sexually transmitted diseases to persist in the absence of other transmission mechanisms. This is the case in many Coleoptera, which suffer from sexually transmitted diseases more than other insect orders (Knell and Webberley, 2004). Modeling infection frequency clines of *Wolbachia* in *D. melanogaster* suggested that adult dormancy during cold periods could be partly responsible for highly variable infection frequencies across the world (Kriesner *et al.*, 2016). Modeling tick-borne pathogen dynamics showed that the major time lags introduced into tick vector development by diapause led to complicated oscillatory patterns of tick-borne encephalitis and Lyme borreliosis disease dynamics in the field (Zhang *et al.*, 2017). Thus, diapause and seasonal cycles are important parameters to consider when assessing host-pathogen dynamics at the population or community scale, especially when planning mitigation for wildlife diseases (Chuche and Thiéry, 2009; Bozzuto and Canessa, 2019).

Ecology and Evolution of Interactions Between Microbes and Diapausing Hosts

A number of laboratory studies on host-microbe interactions have been carried out in model and non-model organisms that, in their natural settings, can undergo diapause. Many of these studies discuss evolutionary and ecological implications without discussing how diapause complicates the understanding of the coevolutionary process. For example, a

study of the effect of the microbiome on the lifespan of the African turquoise killifish draws interesting conclusions about the reciprocal effects of host senescence and microbial community change (Smith *et al.*, 2017); but it does not address at all how microbiome assembly might be established in the wild, where killifish can famously survive complete desiccation of their habitats *via* diapausing eggs (Furness *et al.*, 2015). In fact, diapausing embryonic stages are particularly convenient for researchers studying microbiome functions in the lab, because these stages are inherently stress resistant and thus can be more easily used to generate bacteria-free or gnotobiotic animals for experiments (Nougué *et al.*, 2015; Sison-Mangus *et al.*, 2015). Studies that take this approach should address how the colonization process in these lab experiments is likely to differ from that in natural settings.

As alluded to earlier, it is of interest to understand how host diapause changes the nature of competition between members of an established microbial community. Does diapause relax the selective mechanisms typically imposed by host regulation (Bevins and Salzman, 2011), or have hosts evolved specific mechanisms that act on microbial communities during diapause as part of the suite of changes associated with diapause? The ultimate composition of host-associated microbial communities depends on a combination of “top-down” (*i.e.*, mediated by host physiology and selection on hosts) and “bottom-up” (*i.e.*, mediated by microbial colonization and competition) selective factors (Costello *et al.*, 2012). Given the drastic changes that occur in host physiology during diapause, it has the potential to radically alter the balance of these forces.

From an evolutionary perspective, one of the most important questions to answer about any microbial association is whether it is vertically or horizontally transmitted (or both, known as mixed-mode transmission). By placing offspring far away from their parents and their parental environment in both time and space, embryonic diapause has the potential to profoundly affect opportunities for both vertical and horizontal transmission of microbiota. Furthermore, there has been considerable interest in broad phylogenetic patterns of correlation between host relatedness and microbial community similarity; such patterns can be driven by vertical transmission of microbiomes or by genetic traits of hosts that select for particular bacteria during horizontal transmission (Sanders *et al.*, 2014). Diapausing organisms, especially if they are subject to dispersal during diapause, might have uniquely weakened vertical transmission in addition to repeatedly being exposed to different pools of potential horizontally transmitted microbes.

Predictability of environments, including of biotic interactions, affects evolution of phenotypic plasticity (Whitman and Agrawal, 2009). For many organisms, especially those in which diapause provides opportunities for long-distance dispersal, diapause increases the probability of finding oneself in a completely different microbial milieu than that experienced by one's parents. This is especially important when considering

populations of dormant hosts as biotic reservoirs, because of the possibility of these organisms exiting dormancy only to be exposed to unfamiliar pathogens. Therefore, we might predict that organisms that go through a diapausing stage have a greater range of tolerances for diverse bacteria. In addition, environmental microbes themselves can go through seasonal dynamics, including forming spore banks of resting stages and altering abiotic conditions in a seasonal manner (Lennon and Jones, 2011). It is therefore possible that animal adaptations evolve partly in response to the seasonal dynamics of the environmental bacterial community.

Diapause during embryonic stages can result in a population consisting of discrete, non-overlapping generations. This potentially poses a problem for the long-term persistence of pathogens that depend on these animal hosts. Theory predicts, therefore, that pathogens dependent on hosts that can enter diapause would evolve to be transmitted both horizontally (between unrelated individuals) and vertically (between parents and offspring). In a review on the topic of such mixed-mode transmission, Ebert (2013) cites examples in which a small amount of vertical transmission is sufficient to maintain populations of pathogens that would otherwise go extinct while their host overwinters (Ebert, 2013). Vertically transmitted pathogens are expected to evolve toward lower virulence, because during vertical transmission, pathogen fitness interests are aligned with those of their hosts. Therefore, we would expect pathogens strongly dependent on specific hosts that have the capacity for diapause to be at least occasionally transmitted through diapausing stages and to have minimal fitness costs during this type of transmission. Indeed, one study found that when transmitted vertically into diapausing eggs, the microsporidian parasite *Hamiltosporidium tvarminnensis* does not cause *Daphnia* egg mortality or otherwise influence any fitness parameters but negatively affects host fitness during horizontal transmission (Sheikh-Jabbari *et al.*, 2014). Host diapause may therefore select for phenotypically plastic virulence and transmission strategies in pathogens (Vizoso and Ebert, 2005). Alternatively, it may select for wider host range in pathogens (*i.e.*, the ability to switch to different host species when one host species enters diapause); or it may select for dormancy mechanisms in the pathogen, to allow an inactive environmental reservoir of pathogens to persist when hosts are not available. For example, *Entomophthora* spp., an entomopathogen that attacks numerous species that overwinter as infection-resistant eggs, has the ability to form resting spores (Brandenburg and Kennedy, 1981).

Similar questions about specialization and plasticity apply to beneficial microbiota of diapausing animals. Typically, vertical transmission of microbiomes is ubiquitous, even in the absence of transovarial transmission (Funkhouser and Bordenstein, 2013). Mechanisms such as egg smearing, parental care, or even simple proximity between parents and offspring mean that animals are often more likely to acquire microbiota from their parents than from unrelated individuals. This vertical

transmission leads to co-selection of hosts and microbes, explaining how animals can evolve to depend on unrelated organisms for optimal fitness. However, diapausing embryonic stages may break the chain of transgenerational continuity of microbiota, unless specific mechanisms evolve to preserve it. For example, we might predict that microbes may evolve to coordinate dormancy with the host to persist in association with the dormant embryo. Another possibility is that diapausing hosts have evolved to be able to form beneficial associations anew in every generation, in potentially unfamiliar environments. Specific environmental acquisition is documented in the bean bug *Riptortus pedestris*, a taxon in which both diapause and host-microbe interactions have been studied in mechanistic detail, although connections between these studies have not been made (Ikeno *et al.*, 2011; Kikuchi *et al.*, 2011). *Riptortus pedestris* acquires beneficial *Burkholderia* symbionts efficiently and specifically from soil environments in every new generation (Kikuchi *et al.*, 2011); however, since in this species diapause occurs in adults, not in eggs or embryos, this does not appear to be an example of obligate environmental acquisition resulting from discontinuous generations.

In animals that acquire microbiota from the environment after diapause, we should expect lower dependence of the animal on specific beneficial microbes, because their presence may not be guaranteed. In the absence of specific mechanisms for preserving transmission across diapausing stages, diapausing organisms would be expected to be able to (i) derive benefits from a wide range of bacteria, (ii) have strong genetic mechanisms for selecting beneficial microbes from unfamiliar environments, (iii) be not dependent on bacteria at all, or (iv) be limited in their ecological niche or geographic range by the availability of suitable microbes. In our study of the microbes associated with diapausing eggs of *Daphnia*, we speculated that even if *Daphnia* and its microbes are not strongly evolutionarily linked *via* reliable vertical transmission, they may be ecologically linked by co-dispersal, with the dispersing propagules of the host seeding new environments with microbes originating in the previous environment (Mushegian *et al.*, 2018).

It is important to note that parallels to many of these questions can be found in studies of plants, in which seeds are a recurring, often long-lasting dormant stage and in which some ecologically important interactions with microbial symbionts have been well characterized (Shade *et al.*, 2017). These parallels can inform our understanding of how diapause influences specificity of associations, geographic range, and phenotypic plasticity. All of the scenarios mentioned above have been observed in plants. Some plants transmit symbiotic fungi into their seeds; while these fungi provide important fitness benefits—including, in some cases, being required for germination—they are also sometimes lost from the seeds after long periods of dormancy (Afkhani and Rudgers, 2008). Other plants form symbiotic associations after germination with a wide tax-

onomic range of fungi (Bruns *et al.*, 2002). Still others have taxonomically specialized associations, which nevertheless do not impede the plants' geographic range expansion because the fungi are independently geographically widespread (Davis *et al.*, 2015). Finally, there is evidence that dependence on specific microbial partners, in the absence of reliable transmission through seeds, does in fact limit the geographic range of some plant species (Pringle *et al.*, 2009). It is unknown which of these scenarios are most common in diapausing animals, and we think that examining these questions on a case-by-case basis will yield interesting insights into the opportunities and constraints posed by both dormancy and symbiosis.

Diapause and Host-Microbe Interactions Under Global Change

Global change does not just affect species individually; it also has ripple effects *via* organisms with which a species has interdependencies. It is of general interest to understand how environmental change and host-microbe interactions are linked, and specific questions arise when hosts are able to use dormancy as a key adaptation.

Links between diapause and thermal tolerance are not clearly established in every species (Hodkova and Hodek, 2004), and understanding host-microbe interactions in these species might supply some of these missing connections. Some symbionts are known to alter the physiological properties of holobionts, thus influencing the thermal tolerance of their hosts (Hofmann and Todgham, 2010; Brumin *et al.*, 2011; Corbin *et al.*, 2017; Henry and Colinet, 2018). No studies, to date, have established physiological roles of bacteria in modulating host thermal tolerance specifically during diapause. However, such an effect is plausible, because activation of immune mechanisms can also activate protective mechanisms against abiotic stressors (Jones *et al.*, 2015). An interesting example, from the opposite perspective of cold tolerance, comes from the tick vectors of the human pathogen *Anaplasma phagocytophilum* (Neelakanta *et al.*, 2010). The pathogen induces expression of an antifreeze protein, and correspondingly increases cold tolerance, in the tick. Speculatively, this could be a pathogen-mediated life cycle manipulation that increases the spread of the pathogen during winter.

In addition to directly modulating temperature tolerance, the thermal tolerance range of symbionts themselves can constrain the thermal range of the host. In aphids, for example, the vertically transmitted microbial symbiont *Buchnera* is highly temperature sensitive and thus limits the aphid's geographic distribution (Dunbar *et al.*, 2007). This situation is not uncommon in symbiotic microbes (Webster *et al.*, 2008; Wernegreen, 2012). This is important to consider for diapause evolution in a warming climate because resistance of symbionts to changing temperatures during diapause could determine survival of the host; diapause initiation and timing might

therefore evolve in the context of the preservation of host-microbe relationships. As temperatures change, the balance of stress resistance strategies mediated by dormancy and by microbes may shift.

These issues may be particularly amenable to study in invasive and pest species, where mechanisms of rapid adaptation are of both basic and applied interest. For example, in the bean bug discussed above, diapause timing can affect the seasonal dynamics of crop damage and biological control, while acquisition of insecticide-degrading symbionts mediates insecticide resistance (Kikuchi *et al.*, 2012). It is crucial to understand how such key adaptations interact to determine overall fitness.

The issues raised in *Ecology and Evolution of Interactions Between Microbes and Diapausing Hosts*, above, regarding diapause, microbes, and ecological range are also particularly salient in the context of environmental change. Dispersal and geographic range shifts are important mechanisms of persistence in the face of habitat loss and change, and diapause is frequently a strategy that enables colonization of new habitats. If climate change or environmental degradation also affect the populations of beneficial or detrimental microbes available to infect hosts, then the impact of diapause as a biotic reservoir or dispersal mechanism could potentially be compromised. Considering the important roles of microbiota in animal health, there have recently been calls to explicitly consider disruptions to the microbiome when designing biodiversity conservation and wildlife management practices (Redford *et al.*, 2012; Bahrndorff *et al.*, 2016; Trevelline *et al.*, 2019). Diapause may be a recurring “natural” disruption to microbiota. It is important to understand whether animals require any kind of predictable reassembly of microbiomes after diapause and whether habitat changes or management practices affect these colonization and recolonization dynamics.

Conclusions and Outstanding Questions

Diapause may significantly affect the dynamics of microbes interacting with animals; microbes may impact seasonal strategies in animals and, reciprocally, seasonal environmental change may influence microbial communities within hosts. Currently, both phenomena remain poorly studied. However, without addressing these issues, our understanding of both seasonal adaptation and host-microbe coevolution remains incomplete. Animal-associated microbiota are often a source of unexplained phenotypic variation (Mushegian, 2017), and this applies to experimental studies of diapause as well.

We believe there is ample opportunity for researchers to incorporate microbial perspectives into their studies of diapause and, conversely, to consider diapause as a specific ecological feature that affects the evolution of host-microbe relationships. Microbiome research and diapause research typically take place in different sub-disciplines, with different vocabu-

laries and different approaches to framing questions. We encourage greater mutual attention to work being done in both disciplines, because they raise intersecting questions of ecological and evolutionary importance. Below, we briefly list some research approaches of particular relevance in arthropods.

First, researchers should experimentally investigate how the phenotypes and gene expression patterns of diapause entry and exit are affected by the presence and absence of microbes (either of microbiota in general or of specific infections). These types of studies can take advantage of bacteria-free and gnotobiotic model systems that have been developed in numerous organisms (Dobson *et al.*, 2015; Sison-Mangus *et al.*, 2015; Smith *et al.*, 2017), with the basic experimental design being a comparison of phenotypes in animals with and without exposure to microbes. Given that numerous developmental pathways are sensitive to the presence of microorganisms, disturbances introduced by manipulating the microbial environment might yield insights into the genetic and biochemical basis of diapause even if microbes are not directly involved in ecologically relevant diapause phenotypes in natural settings.

Second, diapausing hosts should be considered a potentially distinct type of environment for microbial communities. How does host dormancy change the survival of the bacteria that make the animal their home? Do any microbes coordinate dormancy with their hosts? How does host dormancy change the arena for competition between bacteria in a host-associated community? Sequencing, culturing, and performing phenotypic and transcriptomic assays of microbiota before, during, and after diapause would provide a foundation for understanding these dynamics.

Third, we encourage more attention to susceptibility to infection, and to specificity of host-microbe associations, in diapausing *versus* non-diapausing organisms. Such studies could be carried out both within species that switch between diapausing and non-diapausing forms and in a comparative framework among closely related species that differ in their diapause strategy. Does diapause change immunological and partner-choice mechanisms in anticipation of unfamiliar microbial interactions? How reliable is vertical transmission of pathogens and mutualists through diapausing immature stages? In the absence of continuity of microbiota across diapausing stages, what environmental and host factors affect re-establishment of associations?

Finally, in a global change context, we encourage attention to the potentially discordant responses of animals and microbes to changing environmental conditions. Insects and their symbionts differ in their thermal tolerance, insect diapause affects seasonal infectious disease dynamics, and diapause and microbial dependency together might place constraints on range expansion and local adaptation. Field studies and common-garden experiments could evaluate how diapause phenotypes and host-microbe associations covary in time and space. Answers to these questions are of both fundamental and applied significance in the face of contemporary rapid climate change.

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