Integrative and Comparative Biology Advance Access published June 6, 2014

Integrative and Comparative Biology

Integrative and Comparative Biology, pp. 1–11
doi:10.1093/icb/icu063

Society for Integrative and Comparative Biology

SYMPOSIUM

From Behavior to Mechanisms: An Integrative Approach to the Manipulation by a Parasitic Fungus (*Ophiocordyceps unilateralis s.l.*) of Its Host Ants (*Camponotus* spp.)

Charissa de Bekker,[1,*] Martha Merrow[†] and David P Hughes[*]

[*]Department of Entomology and Biology, Center for Infectious Disease Dynamics, Pennsylvania State University, University Park, State College, PA 16802, USA; [†]Institute of Medical Psychology, Faculty of Medicine, Ludwig Maximilians Universität München, 80336 Munich, Germany

From the symposium "Parasitic Manipulation of Host Phenotype, or How to Make a Zombie" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 3–7, 2014 at Austin, Texas.

[1]E-mail: c.debekker@psu.edu

Synopsis Co-evolution of parasites and their hosts has led to certain parasites adaptively manipulating the behavior of their hosts. Although the number of examples from different taxa for this phenomenon is growing, the mechanisms underlying parasite-induced manipulation of hosts' behavior are still poorly understood. The development of laboratory infections integrating various disciplines within the life sciences is an important step in that direction. Here, we advocate for such an integrative approach using the parasitic fungi of the genus *Ophiocordyceps* that induce an adaptive biting behavior in *Camponotus* ants as an example. We emphasize the use of behavioral assays under controlled laboratory conditions, the importance of temporal aspects of the behavior (possibly involving the circadian clock), and the need to approach colonizing parasites as organizations with a division of labor.

Introduction

Adaptive manipulation of a host's behavior by parasites occurs when co-evolution between parasites and hosts results in the parasite's ability to alter the host's behavior for its own survival and successful transmission (Moore 2002; Thomas et al. 2005). Much of the evidence for adaptive manipulation of hosts' behavior is inferred from field studies with naturally infected hosts (Moore 2002; Thomas et al. 2005; Libersat et al. 2009; Poulin 2010; Hughes et al. 2012). As important as these studies have been, they have not—and likely cannot—fully elucidated the molecular mechanisms through which parasites accomplish the reported complex manipulations (Thompson and Kavaliers 1994; Klein 2003; Thomas et al. 2005; Lefèvre and Thomas 2008; Libersat et al. 2009; Poulin 2010; Adamo 2013; Hughes 2013). The development of controlled laboratory infections to study behavioral manipulation represents a necessary step toward fully understanding the mechanisms of parasite-induced behavioral changes. As with other approaches to proximate systems (e.g., *Drosophila* studies), controlled studies of parasites that control behavior would allow the reduction of complexity added by environmental influences in the field. Variations in environmental factors such as light, temperature, and time and dose of infection, combined with differences in circumstances at the field site, such as vegetation, predation pressure, and availability of food, lead to individual variations that might obscure the detection of altered behaviors related to parasitic manipulation and to discovery of the compounds and genes involved.

One example in which substantial progress has been made by unraveling compounds involved in change of the rodent-host's behavior in a laboratory setting is the highly prevalent neurotropic parasite *Toxoplasma gondii*. Although this protozoan can infect mammals and birds across the globe, forming slow-growing and persisting neural cysts, it only reproduces in the feline gut. After this stage, infectious

propagules are shed (Dubey and Frenkel 1976; Tenter et al. 2000; Dubey 2009). To complete the parasite's life cycle and switch to a sexual stage, the cat (definitive host) must consume the intermediate host (a rat). Behavioral studies with infected rodents showed that this transmission is likely aided by the loss of the rodent's innate, hard-wired fear of cats' odors (Berdoy et al. 2000; Vyas et al. 2007; Lamberton et al. 2008). Interestingly, even after extensive clearance of parasites, this behavior seems to be sustained in some *T. gondii* strains (Ingram et al. 2013). Analyses looking into the exploratory behavior and social investigation of infected rats showed an increase in risk-taking behavior that is dependent upon the dose and upon the progression of the infection in these animals (Gonzalez et al. 2007). Efforts to unravel how these behavioral changes are established demonstrated that the levels of several compounds are altered in the neurons of infected rodents that contain parasite cysts (Prandovszky et al. 2011; Mitra et al. 2013; Vyas 2013). *Toxoplasma gondii* thus likely uses multiple mechanisms that together change the behavior of rodents.

Another example of a system that is being used to better understand mechanisms underlying behavioral manipulation is the biting behavior in ants that are infected by the fungus *Ophiocordyceps* (Andersen et al. 2009; Hughes et al. 2011a). Fungal parasites within this genus alter the behavior of their ant hosts in ways that facilitate the dispersal of spores. Foraging ants presumably get infected when these spores attach to, and penetrate, their cuticle, after which their body is colonized, as has been shown for related fungal entomopathogens such as *Metarhizium* (Clarkson and Charnley 1996). After the colonization period, the ant abandons its normal activities and leaves the nest. Once outside, the infected ant climbs up the foliage where it latches onto vegetation (Andersen et al. 2009; Pontoppidan et al. 2009). Atrophy of the mandible muscles prevents the animals from falling as they (typically) die hanging upside down from a leaf or twig (Hughes et al. 2011a; C. de Bekker, L. Quevillon, P. B. Smith, K. Fleming, D. Gosh, A. D. Patterson, and D. P. Hughes, submitted for publication). After death, the fungus grows out of the cadaver. It uses its host as a carbon source and as a base for propagation and dissemination of spores (Andersen et al. 2009). The cycle ends with the production of a stroma (stalk) from which sexual spores are transmitted to new ants (Evans 1982; Evans and Samson 1984; Andersen et al. 2009; Hughes et al. 2009). With the recent advancement in methods of isolating and maintaining the fungal parasite, establishing infections, and reconstructing behavioral manipulation under controlled laboratory conditions (C. de Bekker, L. Quevillon, P. B. Smith, K. Fleming, D. Gosh, A. D. Patterson, and D. P. Hughes, submitted for publication), this system provides the exciting opportunity to discover how a fungal parasite can control the behavior of an animal host.

In this opinion piece, we lay out an integrative approach toward unraveling the mechanisms driving behavioral manipulation of *Camponotus* ant species by the fungus *Ophiocordyceps unilateralis sensu lato* (*s.l.*) (Fig. 1). We focus on this system because it offers a useful model to emphasize two aspects that have not been fully considered in previous discussions of parasites manipulating hosts' behavior. The first is that many parasites that control behavior, such as *Toxoplasma* and *Ophiocordyceps*, are single-celled organisms that replicate inside the host. Work on diverse microbial systems is showing how heterogeneity within isogenic populations of cells is important. Here, we wish to emphasize the importance of heterogeneity for studies of behavioral manipulation. A second focus we wish to encourage is that of circadian rhythms. A large body of work is highlighting the molecular basis of clocks in different organisms. Since we know many manipulative behaviors are highly synchronized we advocate a greater focus on the biology of 24 h rhythms when studying parasites that control behavior.

Understanding how behavioral manipulation occurs is necessarily a highly interdisciplinary endeavor since this subject spans various disciplines within the life sciences, ranging from natural history, evolution, and behavioral ecology to genetics, cell biology, and biochemistry. This means that each of these fields taken in isolation is not powerful enough to obtain a complete understanding of parasite-induced behavioral manipulation. We therefore advocate combining techniques ranging from behavioral ecology to molecular "-omics" tools, to bring us closer to understanding this phenomenon. Here, we discuss how we use controlled laboratory infections, followed by behavioral assays, to study how ants' behavior is affected by *O. unilateralis s.l.* and as a basis for collecting samples for "-omics" studies to discover the genes and compounds involved. When studying parasite-induced behavioral manipulation, the host's behavior functions as a read out for the parasite's success and progression, making controlled behavioral assays very important. From this basis, we then stress the importance of incorporating concepts of monoclonal heterogeneity and chronobiology into these experiments. Not only do field and laboratory studies both suggest that 24 h cycles are necessary for

establishing *O. unilateralis*' manipulation of ants (Hughes et al. 2011a; C. de Bekker, L. Quevillon, P. B. Smith, K. Fleming, D. Gosh, A. D. Patterson, and D. P. Hughes, submitted for publication), but other literature also demonstrates involvement of biological clocks in other parasite–host interactions. Furthermore, we introduce the molecular biological concept of heterogeneity within a monoclonal population to suggest that the samples put toward "-omics" studies to unravel the molecular mechanisms underlying manipulation of ants' behavior should be used within this framework. There is an increasing amount of evidence that populations of isogenic cells are heterogeneous and display a certain "division of labor." This means that a fungal population inside an ant host will react differently to the different tissues it encounters when growing inside the ant's body. Therefore, when attempting to determine the complex mechanisms underlying parasite-induced behavioral manipulation, the complex interactions between parasite and the different tissues of the host should be taken into account.

Behavioral assays following laboratory infections

To elucidate the molecular mechanisms underlying parasite-induced behavioral manipulation and linking them to their phenotypic impacts, sampling relies on the behavioral patterns we observe upon infection. The ability of a parasite to modify its host's phenotype in terms of behavior is the product of natural selection acting on the genes of the parasite (Thomas et al. 2005; Cézilly et al. 2010). The manipulated behavior we observe is therefore an extended phenotype of the parasite (Dawkins 1982): The expression of the parasite's genes changes the expression of the host's genes resulting in an altered behavior at the expense of the host's fitness but favoring the transmission of the parasite's genes. To be able to study what genes and molecules of the parasite are involved in parasite-induced behavioral manipulation, or how the host is affected at the molecular level for that matter, we thus rely on sampling based on this extended phenotype-framework. The extended phenotype comprises the expression of behavioral traits that are different in an infected individual versus a control, or the significant correlation between expression of a trait and level of infection (Moore 2002). A suite of interrelated traits of the host are targeted (Cézilly and Perrot-Minnot 2010; Thomas et al. 2010), often resulting in manipulated hosts that are radically different in several phenotypic dimensions compared with non-parasitized hosts (Fig. 1).

Biological processes such as gene expression and behavior are also impacted by changes in environmental cues. This could make the use of sensitive "-omics" tools such as RNASeq on samples obtained from a natural environment rather tricky. Although successful "-omics" studies exist, such as the proteomics performed on Gordian worms that induce their cricket hosts to jump into water where the

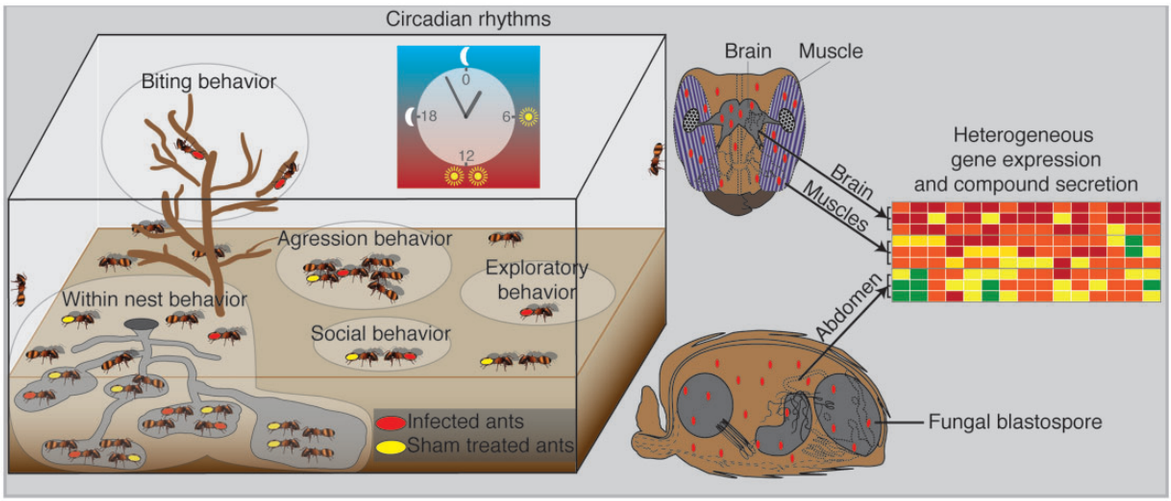

Fig. 1 Summary of the integrative approach toward unraveling the molecular mechanisms driving behavioral manipulation of *Camponotus* ants by *Ophiocordyceps unilateralis s.l.* by means of controlled infections in the laboratory. In these experiments, observations are made by scoring for several behavioral traits. Environmental conditions are maintained constant with strict 24-h (circadian) rhythms for light and temperature. To discover the genes and compounds involved in the observed manipulations, specific interactions of the parasite with the host's tissues are analyzed separately.

worm can exit and reproduce (Biron et al. 2005a, 2005b, 2005c; Biron and Loxdale 2013), stochastic noise introduced by both biotic and abiotic factors could obscure the differential expression of "manipulator substances" of interest, or be mistaken for them. Controlled laboratory studies in which infection and manipulation are successfully reconstructed provide a great solution to this problem. An example of a system in which a tremendous amount of progress has been made this way is that of the jewel wasp (*Ampulex compressa*) that injects venom into the brain of the American cockroach (*Periplaneta Americana*). The venom takes away the roach's motivation to initiate locomotion (Libersat and Gal 2013). To reduce noise and increase replicability between infection studies coupled to behavioral assays, we should aim to control as many factors as possible, for example, using incubators, standard food sources, and controlled light conditions. This will increase the possibility that the differences measured in gene expression or compound levels are indeed related to parasite-induced manipulation and not to stochasticity.

When we translate this to a setup in which to sample *Camponotus* that are infected and manipulated by *O. unilateralis s.l.*, based on observed changes in behavior, there are some basic features of an ideal protocol. We aim to infect individuals with a fixed dose of fungal material by injection (C. de Bekker, L. Quevillon, P. B. Smith, K. Fleming, D. Gosh, A. D. Patterson, and D. P. Hughes, submitted for publication) because ants groom each other as part of their social immunity (Schmid-Hempel 1998) and may thus clean spores away. Furthermore, sham treatments should be introduced as controls to ensure that the altered behavior observed is due to the parasite and is not an artifact of the treatment (C. de Bekker, L. Quevillon, P. B. Smith, K. Fleming, D. Gosh, A. D. Patterson, and D. P. Hughes, submitted for publication). All experimental replicates should have the same number of infected, sham-treated, and untreated individuals because the number of individuals can influence the colony's dynamics (Gordon 1987, 1989) and therefore individual behaviors. Furthermore, similar environments should be provided with equal sizes of cages and with controlled temperature, light, and humidity cycles (Fig. 1). Although the death grip of ants infected with *O. unilateralis s.l.* is easily recognizable, such a standardized protocol will allow the discovery of more subtle changes in behavioral patterns as the infection progresses than has heretofore been possible.

Quantifiable behavioral changes as an effect of infection by parasites can also be exclusive of an adaptive manipulation. Pathological by-products of infection may affect hosts' behavior, sometimes causing difficulties in distinguishing illness and true manipulation (Poulin 1995). By comparing suspected behavioral manipulation via a particular parasitic infection with behavior of animals infected with related generalist species, true adaptive parasitic manipulation may be recognized. Inclusion of strains of the related generalist fungal species *Beauveria bassiana* and *Metarhizium brunneum* for comparison with results from infection with *O. unilateralis s.l.* could therefore be very informative. Similarly, the comparison of the ability of one parasite to manipulate the behavior of multiple host species can also be very powerful. Because parasite-induced manipulation of hosts' behavior is shaped by co-evolution between host and parasite, often this has resulted in species-specificity. The manipulated behavior of ants can, for instance, be traced all the way back to the Eocene (Hughes et al. 2011b) and as such has resulted in a high specificity with each infected species of ant examined being infected by its own species of *Ophiocordyceps* fungus (Evans et al. 2011a, 2011b; Kepler et al. 2011). Moreover, not all sympatric species of ants that are ecologically and phylogenetically similar are necessarily found to be infected (Evans 1974, 1982; Sanjuán et al. 2001; Andersen et al. 2009; Pontoppidan et al. 2009) and even when artificially infected in the laboratory, only those species that are found to be manipulated in nature display the characteristic manipulated biting behavior (C. de Bekker, L. Quevillon, P. B. Smith, K. Fleming, D. Gosh, A. D. Patterson, and D. P. Hughes, submitted for publication). This suggests that there are barriers to successful infection and manipulation, making examination of parasites' intraspecific variability in manipulating the behavior of their various species of host very informative at both the behavioral and molecular level. In fact, a recent *ex vivo* study has shown that *O. unilateralis* reacts differently to various ant species' brains by secreting a different array of metabolites (C. de Bekker, L. Quevillon, P. B. Smith, K. Fleming, D. Gosh, A. D. Patterson, and D. P. Hughes, submitted for publication). This suggests that the ability, or inability, to manipulate the behavior of various ant species might lie at metabolite level.

Circadian rhythms in parasite and host

Living organisms are exposed to highly predictable rhythms of light and temperature each day (e.g.,

Wagner-Smith and Kay 2000; Bell-Pedersen et al. 2005; Johnson et al. 2011; Buhr and Takahashi 2013). These changes in the physical environment represent a stress for living systems since, for instance, basic biochemical rates will change with temperature, and exposure to UV light can damage DNA. The biological answer to regular, daily environmental oscillations is the circadian clock. The clock is a temporal program that serves to "bin" certain functions to specific times of day. The clock acts on the level of the cell but complex organisms possess a remarkable circadian organization that build up from cells to organs to circuits to behavior (Kramer and Merrow 2013). A key component of the molecular mechanisms of the circadian clock is a network of genes encoding a negative transcriptional–translational feedback loop (Roenneberg and Merrow 2003; Buhr and Takahashi 2013). Including 24 h zeitgeber (i.e., synchronizer) cycles into controlled laboratory studies may thus be essential for their outcome.

Circadian clocks are observed in organisms from all phyla; thus, we are all surrounded by a biota permeated with distinct chronobiological behaviors. Mates, food sources, predators, and parasites are also exposed to periodic changes and likely will have circadian clocks. In social insects, the role circadian clocks play in their behavior has been extensively studied in honeybees (Bloch 2010) and also in several ant species such as *Camponotus compressus*, *Camponotus paria* (Sharma et al. 2004; Lone et al. 2010), and *Solenopsis invicta* (Ingram et al. 2012). When interested in studying "normal" ant behavior in the laboratory, with the goal of comparing this to manipulated behavior induced by a fungal parasite, it is of great importance to perform experiments under strict 24 h zeitgeber cycles. Colonies of social insects, such as ants, generally display a division of labor, showing a number of tasks that are exquisitely synchronized (Bourke and Franks 1995). It appears that the timing of daily behavior of social insects, such as ant species *C. compressus*, shows plasticity. Ants are a certain "chronotype" according to caste, meaning that the timing of certain tasks within the colony is caste-specific (Sharma et al. 2004). Similarly, circadian clocks in *Drosophila melanogaster* were shown to be developmentally plastic with circadian patterns changing between certain life stages (Sharma 2003). A change in chronotype over lifetime is seen even in humans (Roenneberg et al. 2004). Finally, the clock is remarkably sensitive to light, widely thought to be the most important zeitgeber that acts to synchronize or to entrain the circadian clock to the 24 h day. Thus, chronotype (the timing of sleeping and waking) changes with the seasons or with where one lives within a time zone (Kantermann et al. 2007).

Plasticity of animal behavior is important for survival. However, it also represents an Achilles heel, since plasticity is a prerequisite for manipulation (Adamo 2002). The systematic entrainment properties of the daily temporal program might therefore be a hallmark for manipulating parasites to change the timing of certain behaviors or physiological aspects of their hosts for their own benefits. Although the evidence for this is still only indirect, the so-called "tree top disease" fits this hypothesis. Baculoviruses are known to introduce this disease in their caterpillar hosts (Hofman 1891). Just before death, infected larvae of the gypsy moth *Lymantria dispar* climb to the top of their hosts' trees where they die, liquefy, and release infective virus particles (D'Amico and Elkinton 1995). In contrast, healthy individuals display a daily periodic behavior in which they climb up onto the leaves to feed at night after which they climb back down to the soil and avoid predation by birds during the day. A recent study using several baculovirus constructs showed that a single viral gene is responsible for the inactivation of molting hormone 20-hydroxyecdysone in infected caterpillars, resulting in disruption of their climbing behavior (Hoover et al. 2011). Ecdysteroid synthesis in insects is normally under circadian regulation by a photosensitive oscillator (Vafopoulou and Steel 1998) and is of central importance in the regulation of behavior (Richter et al. 1989) and development (Gilbert et al. 1997) in insects. The disruption of an otherwise periodic behavior regulated by an oscillator synchronized by light, thus suggests that this baculovirus has found a way to break into a clock that is of great importance to the survival of the host.

Foraging worker ants form the caste that is generally found infected by the manipulating fungus *O. unilateralis s.l.* Their foraging behavior, which is under clock control, is notably disrupted when the infected ant abandons its activities as a worker in the colony, climbs up onto vegetation, latches on by its mandibles, and dies. This implies the parasite might be breaking into the clock here as well, changing the expression of genes that are of importance for establishing certain behaviors. The manipulated behavior itself also appears to be highly synchronized. In Thailand, infected *Camponotus* ants were found to bite foliage at about solar noon after which death followed at about sunset (Hughes et al. 2011a). Similarly, synchronization was found in experiments performed in the laboratory with *Camponotus* and

O. unilateralis s.l. from North America (C. de Bekker, L. Quevillon, P. B. Smith, K. Fleming, D. Gosh, A. D. Patterson, and D. P. Hughes, submitted for publication). Laboratory-infected ants displaying the characteristic biting behavior were always found in the morning, followed by death in the early afternoon. Moreover, this manipulation has only been observed within the third week post infection. Noteworthy is the fact that the behavioral manipulation has only been successfully achieved in the laboratory upon the introduction of fixed 24-h light and temperature cycles (Fig. 1). Manipulated behavior appears to be synchronized in other systems as well. Another example is that of parasitic trematodes from the genus *Dicrocoelium* that cycle in land molluscs and ants, and have numerous mammals that act as their definitive hosts across several continents (Malek 1980). In this complex parasitic life cycle (Krull and Mapes 1952, 1953), the behavior of the intermediate ant host is changed to favor ingestion by the definitive host. Infected ants temporarily attach themselves to grass, predominantly at nightfall, and thereby promote ingestion by the grazing host (Spindler et al. 1986). This behavior appears to be synchronized by temperature. Lower temperatures lead to an increased amount of infected ants found attached to grass, whereas the number decreases at higher temperatures (Badie et al. 1973; Manga-González et al. 2001). Synchronization of the manipulated host's behavior indicates either disruption of daily timing of certain genes in the ant by the parasite, time-of-day specific actions directed by the fungal clock, or both.

Alternatively, the parasite has to adapt to the host's clock and uses a completely different strategy to change the host's behavior. Regardless, the incorporation of chronobiological principles in controlled laboratory experiments will still be of great importance as the host's behavior and the parasite's fitness both rely on periodicity. Malaria (*Plasmodium*) parasites provide an example of the latter. These parasites replicate asexually in a vertebrate host and sexually in the mosquito vector. At the end of the cell cycle, during the night, mature parasites synchronously release multiple progeny. In addition, species that infect humans have synchronous durations of cell-cycle that cause recurrent fever, which is so precise that it is used to diagnose the disease (Garcia et al. 2001). The periodicity is always a multiple of 24 h, suggesting that the parasite's rhythms are either regulated by circadian clocks or that its cell cycle is gated by them. The disruption of these rhythms results in a decrease in replication and transmission (O'Donnell et al. 2011). Furthermore, it appears that a mismatch to the host's circadian rhythms results in parasites that cause less anaemia and are therefore less virulent to their hosts (O'Donnell et al. 2013). In addition, there is a growing evidence for the rhythmic regulation by the clock of defense genes in hosts and the involvement of clock genes in parasites' virulence. Disruption of circadian rhythms in animals can lead to increased susceptibility to pathogens (Lee and Edery 2008; Castanon-Cervantes et al. 2010) and even the control of plants' defense genes by circadian clocks has been suggested (Wang et al. 2011). On the other hand, light also appears to be an important regulator of fungal pathogenesis (Idnurm and Crosson 2009). Involvement of orthologs of the well-studied WC-1 (from *white collar-1*), a blue-light receptor that regulates the circadian clock and spore-formation in the fungus *Neurospora crassa* (Ballario et al. 1996; Linden et al. 1997), has been demonstrated to modulate the virulence of a pathogen of humans, *Cryptococcus neoformans* (Idnurm and Heitman 2005) and of rice blast fungus, *Magnaporthe oryzae* (Kim et al. 2011). Furthermore, it has been shown for a necrotrophic plant pathogen, *Botrytis cinerea*, that the White Collar Complex, of which WC-1 is part, is needed for tolerating excessive illumination as well as for achieving full virulence in the presence of light (Canessa et al. 2013).

Taken together, this all suggests that circadian rhythms are of great importance to parasite–host interactions and to animal behavior. Although evidence for the change of rhythmic regulation of genes involved in hosts' behavior through the disruption of daily timing by a manipulating parasite is still indirect, certain manipulated behaviors do appear to be very synchronized as well. This indicates that chronobiological concepts should be taken into account when laboratory infections and behavioral assays are used to unravel the behavioral manipulative mechanisms of parasites.

Heterogeneous parasite–host interactions

When the aim is to study the mechanisms by which parasites control hosts' behavior, it is necessary to be cognizant of the heterogeneous nature of the parasite. This is especially true for single-celled parasites that replicate within the host. A parasite entering a host and colonizing it encounters different environments in the form of various host tissues and an activated immune system. To be able to successfully deal with such a heterogeneous environment, to overcome the immune system, to progress the

infection and move toward transmission, both gene expression and compound secretion of monoclonal parasites can be expected to be highly dynamic. Despite this, colonizing monoclonal parasites are not generally considered as having a division of labor. This concept of heterogeneity is, however, important to the discovery of the mechanisms underlying parasites' behavioral manipulation, and should therefore also be incorporated into studies that have this aim. One example of a parasite with an unexpected heterogeneous organization is the trematode flatworm belonging to the *Himasthla* sp. B (HIMB) that infects the California horn snail, *Cerithidea californica* (Haldeman 1840). These trematodes undergo repeated clonal reproduction within their molluscan hosts (Galaktionov and Dobrovolskij 2003), forming colonies and blocking the host's reproduction (Hechinger et al. 2009). Recent research has shown that this clonal reproduction results in colonies with specialized soldier and reproductive castes that display a clear division of labor (Hechinger et al. 2011). Soldiers do not reproduce and appear to be morphologically different from the reproductive castes. Furthermore, soldiers are more active and are disproportionately common in areas of host invasion where only this particular caste attacks invaders of other colonies. To study gene expression in this trematode and how that is related to invasion avoidance, castes from a host invasion area should therefore be studied separately rather than by extracting RNA from the trematode colony inside the snail as a whole. To be able to study the mechanisms of, in this case, invasion avoidance, the heterogeneous host–parasite interactions should thus be taken into account. Failing to do so and taking the trematode population as a whole to study gene expression, means that one is looking at a mere intermediate gene expression throughout the colony and therefore might not find the genes that are up-regulated or down-regulated in the soldier's caste during invasion.

Heterogeneity within an isogenic population also exists in the microbial world. Clonal cultures of bacteria such as *Escherichia coli* and *Bacillus subtilis* exhibit phenotypic variation related to responses to environmental stress (Elowitz et al. 2002; Veening et al. 2008a), suggesting that this heterogeneity aids in the survival of cells under adverse conditions (Veening et al. 2008b). The fungal pathogen *Candida albicans*, which infects humans, forms a heterogeneous biofilm that contains persister cells. These cells comprise a small fraction of the population and are, with their low cellular activity, tolerant to stress, thereby surviving challenges that are lethal to growing cells (Nobile and Mitchell 2007). Heterogeneity can even be found in fungal colonies that are not parasites. It has been shown that yeast (*Saccharomyces cerevisiae*) can change patterns in cell shape and cell division during starvation and form pseudohyphae that explore for nutrients (Gimeno et al. 1992). Furthermore, research on the saprophytic mold *Aspergillus niger* has shown that fungal growth in a simple medium displays heterogeneous gene expression at different levels: between neighboring fungal cells; between different zones within a colony growing in a Petri dish; and between microcolonies growing in liquid shaken cultures (Levin et al. 2007; de Bekker et al. 2010, 2011; Vinck et al. 2011).

Heterogeneity may be less for macro-parasites, such as the previously mentioned Gordian worm. This worm, which can be many times longer than its insect host, resides in the abdomen, never migrating to the brain. As such extraction of proteins from the parasite as a whole has led to insights into the mechanisms it uses to induce the suicidal behavior seen in its hosts (Biron et al. 2005b). Nevertheless, these studies have shown a temporal heterogeneity, with differences in the presence and concentration of protein before, during, and after manipulation. Furthermore, the central nervous system (CNS) of the host is the part of the host that is being affected, making the concept of heterogeneity still relevant when observed from the host's perspective (Biron et al. 2005b). It is also noteworthy that these studies have been conducted within the framework of circadian rhythms, as advocated above, since healthy insect controls were collected at the same times as the infected individuals.

So, how does this translate to our example of *O. unilateralis s.l.* changing the behavior of *Camponotus* ants? Evidence suggests that parasites that can adaptively manipulate a host to display a novel behavior secrete compounds that act directly on the host's CNS (Adamo 2013). Since the biting behavior observed in ants infected with *Ophiocordyceps* is such a striking behavior, we thus expect the fungal cells residing right beside the brain to directly attack the CNS. In addition, the examination of the mandibular muscles of an infected and recently killed *Camponotus* ant revealed fungal cells within atrophied muscle tissue (perhaps an indication of the different levels to which reference was made above) (Hughes et al. 2011a). A metabolomics study in which *O. unilateralis s.l.* was grown *ex vivo* in the presence of ant brains and mandible muscles revealed that this fungus indeed reacts heterogeneously to these tissues by secreting a different array of

metabolites (C. de Bekker, L. Quevillon, P. B. Smith, K. Fleming, D. Gosh, A. D. Patterson, and D. P. Hughes, submitted for publication) (Fig. 1). A similar study using strains of the related species *B. bassiana* and *M. brunneum*, that do not change their hosts' behavior, led to the same conclusions (de Bekker et al. 2013). This demonstrates that these fungal parasites, even when they do not adaptively change the behavior of their hosts, display a division of labor between cells within the colony that deal with the diverse conditions they encounter inside their host. *In vivo* "-omics" studies following controlled laboratory infections coupled with behavioral assays to investigate the mechanisms underlying fungal manipulation of the ant host's behavior could thus greatly benefit from separating different tissues of the host or, at least, different body parts.

To be able to unravel the molecular mechanisms that isogenic parasite populations employ in changing the behavior of their hosts, the heterogeneous interactions with different host tissues must be considered. Investigating only the cells that are specifically involved in the parasite–host interactions relevant to our scientific questions will better reveal the compounds and genes involved in those interactions while failing to do so might lead to measuring a mere intermediate of all specific molecular mechanisms together. Novel techniques such as metabolomics on specific *ex vivo* host tissue–parasite interactions appear to have great potential to reveal the metabolites involved. Furthermore, state-of-the-art techniques such as laser capture microdissection to sample cells of interest from *in vivo* infection experiments for RNAseq could aid in the discovery of the genetic basis underlying the extended phenotype of parasitic behavioral manipulation.

Conclusion

Adaptive manipulation of animal behavior by parasites spans many disciplines within the life sciences such as behavioral ecology, evolution, neurobiology, chronobiology, and molecular biology. The field is moving from important descriptive natural-history studies into elucidating the mechanisms underlying the manipulation of one organism by another. The complexity of this phenomenon asks for an integrative approach in which different biological frameworks from various disciplines are combined. Here, we advocate that controlled laboratory infections, tied in with behavioral studies assessing changed behavior in which chronobiological concepts are incorporated, will lead to samples suitable for various "omics" studies. Approaching these samples within the concept of heterogeneous parasite–host interactions will elucidate the genes and compounds involved in the manipulation of behavior observed in the parasite.

Acknowledgment

The authors would like to thank Roel Fleuren for helping us to create a figure that illustrates the integrative approach discussed in this article.

Funding

This work was supported by the Society for Integrative and Comparative Biology (Division of Invertebrate Biology, Division of Animal Behavior, and Division of Neurobiology); The American Microscopical Society; the National Science Foundation [IOS 1338574]; and Marie Curie Actions [IOF 299501].

References

Adamo SA. 2002. Modulating the modulators: parasites, neuromodulators and host behavioral change. Brain Behav Evol 60:370–7.

Adamo SA. 2013. Parasites: evolution's neurobiologists. J Exp Biol 216:3–10.

Andersen SB, Gerritsma S, Yusah KM, Mayntz D, Hywel-Jones NL, Billen J, Boomsma JJ, Hughes DP. 2009. The life of a dead ant: the expression of an adaptive extended phenotype. Am Nat 174:424–33.

Badie A, Vincent M, Morel-Vareille C, Rondelaud D. 1973. Cycle de *Dicrocoelium dendriticum* (Rudolphi, 1819) en Limousin. Ethologie des fourmis parasitées par les métacercaires. C R Séances Soc Biol 167:725–7.

Ballario P, Vittorioso P, Magrelli A, Talora C, Cabibbo A, Macino G. 1996. White collar-1, a central regulator of blue light responses in *Neurosopora*, is a zinc finger protein. EMBO J 15:1650–7.

Bell-Pedersen D, Cassone VM, Earnest DJ, Golden SS, Hardin PE, Thomas TL, Zoran MJ. 2005. Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat Rev Genet 6:544–56.

Berdoy M, Webster JP, Macdonald DW. 2000. Fatal attraction in rats infected with *Toxoplasma gondii*. Proc R Soc B 267:1591–4.

Biron DG, Joly C, Galéotti N, Ponton F, Marché L. 2005a. The proteomics: a new prospect for studying parasitic manipulation. Behav Process 68:249–53.

Biron DG, Loxdale HD. 2013. Host–parasite molecular cross-talk during the manipulative process of a host by its parasite. J Exp Biol 216:148–60.

Biron DG, Marché L, Ponton F, Loxdale HD, Galéotti N, Renault L, Joly C, Thomas F. 2005b. Behavioural manipulation in a grasshopper harbouring hairworm: a proteomics approach. Proc Biol Sci 272:2117–26.

Biron DG, Moura H, Marché L, Hughes AL, Thomas F. 2005c. Towards a new conceptual approach to "parasito-proteomics". Trends Parasitol 21:162–8.

Bloch G. 2010. The social clock of the honeybee. J Biol Rhythms 25:307–17.
Bourke AF, Franks NR. 1995. Social evolution in ants. Princeton (NJ): Princeton University Press. p. 400–43.
Buhr ED, Takahashi JS. 2013. Molecular components of the mammalian circadian clock. In: Kramer K, Merrow M, editors. Circadian clocks. Berlin: Springer. p. 3–27.
Canessa P, Schumacher J, Hevia MA, Tudzynski P, Larrondo LF. 2013. Assessing the effects of light on differentiation and virulence of the plant pathogen *Botrytis cinerea*: Characterization of the *White Collar* Complex. PLoS One 8:e84223.
Castanon-Cervantes O, Wu M, Ehlen JC, Paul K, Gamble KL, Johnson RL, Besing RC, Menaker M, Gewirtz AT, Davidson AJ. 2010. Dysregulation of inflammatory responses by chronic circadian disruption. J Immunol 185:5796–805.
Cézilly F, Perrot-Minnot M-J. 2010. Interpreting multidimensionality in parasite-induced phenotypic alterations: panselectionism versus parsimony. Oikos 119:1224–9.
Cézilly F, Thomas F, Médoc V, Perrot-Minnot M-J. 2010. Host-manipulation by parasites with complex life cycles: adaptive or not? Trends Parasitol 26:311–7.
Clarkson JM, Charnley AK. 1996. New insights into the mechanisms of fungal pathogenesis in insects. Trends Microbiol 4:197–203.
D'Amico V, Elkinton JS. 1995. Rainfal effects on transmission of gypsy-moth (Lepidoptera, Lymantriidae) nuclear polyhedrosis-virus. Environ Entomol 24:1144–9.
Dawkins R. 1982. The extended phenotype. New York: Oxford University Press.
de Bekker C, Bruning O, Jonker MJ, Breit TM, Wösten HAB. 2011. Single cell transcriptomics of neighboring hyphae of *Aspergillus niger*. Genome Biol 12:R71.
de Bekker C, Smith PB, Patterson AD, Hughes DP. 2013. Metabolomics reveals the heterogeneous secretome of two entomopathogenic fungi to *ex vivo* cultured insect tissues. PLoS One 8:e70609.
de Bekker C, van Veluw GJ, Vinck A, Wiebenga LA, Wösten HAB. 2010. Heterogeneity of *Aspergillus niger* microcolonies in liquid shaken cultures. Appl Environ Microbiol 77:1263–7.
Dubey JP. 2009. History of the discovery of the life cycle of *Toxoplasma gondii*. Int J Parasitol 39:877–82.
Dubey JP, Frenkel JK. 1976. Feline toxoplasmosis from actutely infected mice and development of *Toxoplasma* cysts. J Protozool 23:537–46.
Elowitz MB, Levine AJ, Siggia ED, Swain PS. 2002. Stochastic gene expression in a single cell. Science 297:1183–6.
Evans HC. 1974. Natural control of arthropods, with special reference to ants (Formicidae), by fungi in tropical high forest of Ghana. J Appl Ecol 11:37–49.
Evans HC. 1982. Entomogenous fungi in tropical forest ecosystems—an appraisal. Ecol Entomol 7:47–60.
Evans HC, Elliot SL, Hughes DP. 2011a. Hidden diversity behind the zombie-ant fungus *Ophiocordyceps unilateralis*: four new species described from Carpenter ants in Minas Gerais, Brazil. PLoS One 6:e17024.
Evans HC, Elliot SL, Hughes DP. 2011b. *Ophiocordyceps unilateralis*: a keystone species for unraveling ecosystem functioning and biodiversity of fungi in tropical forests? Commun Integr Biol 4:598–602.
Evans HC, Samson RA. 1984. *Cordyceps* species and their anamorphs pathogenic on ants (Formicidae) in tropical forest ecosystems. 2. The *Camponotus* (Formicinae) complex. Trans Br Mycol Soc 82:127–50.
Galaktionov K, Dobrovolskij A. 2003. The biology and evolution of trematodes: an assay on the biology, morphology, life cycles, transmission, and evolution of digenetic trematodes. DordrechtKluwer Academic Publishers.
Garcia CRS, Markus RP, Madeira L. 2001. Tertian and quartan fevers: temporal regulation in malarial infection. J Biol Rhythms 16:436–43.
Gilbert LI, Song Q, Rybczynski R. 1997. Control of ecdysteroidogenesis: activation and inhibition of prothoracic gland activity. Invert Neurosci 3:205–16.
Gimeno CJ, Ljungdahl PO, Styles CA, Fink GR. 1992. Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and *RAS*. Cell 68:1077–90.
Gonzalez LE, Rojnik B, Urrea F, Urdaneta H, Petrosino P, Colasante C, Pino S, Hernandez L. 2007. *Toxoplasma gondii* infection lower anxiety as measured in the plus-maze and social interaction tests in rats. A behavioral analysis. Behav Brain Res 177:70–9.
Gordon DM. 1987. Group-level dynamics in harvester ants: young colonies and the role of patrolling. Anim Behav 35:833–43.
Gordon DM. 1989. Dynamics of task switching in harvester ants. Anim Behav 38:194–204.
Haldeman S. 1840. A monograph of the Limniades and other freshwater univalve shells of North America. Philadelphia. https://archive.org/details/monographoflimni00hald.
Hechinger RF, Lafferty KD, Mancini FT III, Warner RR, Kuris AM. 2009. How large is the hand in the puppet? Ecological and evolutionary factors affecting body mass of 15 trematode parasitic castrators in their snail host. Evol Ecol 23:651–67.
Hechinger RF, Wood AC, Kuris AM. 2011. Social organization in a flatworm: trematode parasites form soldier and reproductive castes. Proc R Soc B 278:656–5.
Hofman O. 1891. Insektenötende pilze mit besonderer berücksichtigung der nonne. Weber, Frankfurt.
Hoover K, Grove M, Gardner M, Hughes DP, McNeil J, Slavicek J. 2011. A gene for an extended phenotype. Science 333:1401.
Hughes DP. 2013. Pathways to understanding the extended phenotype of parasites in their hosts. J Exp Biol 216:142–7.
Hughes DP, Andersen SB, Hywel-Jones NL, Himaman W, Billen J, Boomsma JJ. 2011a. Behavioral mechanisms and morphological symptoms of zombie ants dying from fungal infection. BMC Ecol 11:13.
Hughes DP, Brodeur J, Thomas F. 2012. Host manipulation by parasites. Oxford: Oxford University Press.
Hughes DP, Evans HC, Hywel-Jones NL, Boomsma JJ, Armitage SAO. 2009. Novel fungal disease in complex leaf-cutting ant societies. Ecol Entomol 34:214–20.
Hughes DP, Wappler T, Labandeira CC. 2011b. Ancient death-grip leaf scars reveal ant-fungal parasitism. Biol Lett 7:67–70.

Idnurm A, Crosson S. 2009. The photobiology of microbial pathogenesis. PLoS Pathog 5:e1000470.

Idnurm A, Heitman J. 2005. Light controls growth and development via a conserved pathway in the fungal kingdom. PLoS Biol 3:e95.

Ingram KK, Kutowoi A, Wurm Y, Shoemaker D, Meier R, Bloch G. 2012. The molecular clockwork of the fire ant *Solenopsis invicta*. PLoS One 7:e45715.

Ingram WM, Goodrich LM, Robey EA, Eisen MB. 2013. Mice infected with low-virulence strains of *Toxoplasma gondii* lose their innate aversion to cat urine, even after extensive parasite clearance. PLoS One 8:e75246.

Johnson CH, Stewart PL, Egli M. 2011. The cyanobacterial circadian system: from biophysics to bioevolution. Annu Rev Biophys 40:143–67.

Kantermann T, Juda M, Merrow M, Roenneberg T. 2007. The human circadian clock's seasonal adjustment is disrupted by daylight saving time. Curr Biol 17:1996–2000.

Kepler RM, Kaitsu Y, Tanaka E, Shimano S, Spatafora JW. 2011. *Ophiocordyceps pulvinata* sp. nov., a pathogen of ants with a reduced stroma. Mycoscience 52:39–47.

Kim S, Singh P, Park J, Park S, Friedman A, Zeng T, Lee YH, Lee K. 2011. Genetic and molecular characterization of a blue light photoreceptor MGWC-1 in *Magnaporthe oryzae*. Fungal Genet Biol 48:400–7.

Klein SL. 2003. Parasite manipulation of the proximate mechanisms that mediate social behavior in vertebrates. Physiol Behav 79:441–9.

Kramer A, Merrow M. 2013. Circadian clocks. Berlin: Springer.

Krull WH, Mapes CR. 1952. Studeis on the biology of *Dicrocoelium dendriticum* (Rudolphi, 1819) Looss, 1899 (Trematoda: Dicrocoeliidae), including its relation to the intermediate host, *Cionella lubrica* (Müller). IX. Notes on the cyst, metacercaria, and infection in the ant, *Formica fusca*. Cornell Vet 42:603–4.

Krull WH, Mapes CR. 1953. Studeis on the biology of *Dicrocoelium dendriticum* (Rudolphi, 1819) Looss, 1899 (Trematoda: Dicrocoeliidae), including its relation to the intermediate host, *Cionella lubrica* (Müller). IX. Notes on the cyst, metacercaria, and infection in the ant, *Formica fusca*. Cornell Vet 43:389–410.

Lamberton PHL, Donnelly CA, Webster JP. 2008. Specificity of the *Toxoplasma gondii*-altered behaviour to definitive versus non-definitive host predation risk. Parasitology 135:1143–50.

Lee J-E, Edery I. 2008. Circadian regulation in the ability of *Drosophila* to combat pathogenic infections. Curr Biol 18:195–9.

Lefèvre T, Thomas F. 2008. Behind the scene, something else is pulling the strings: emphasizing parasitic manipulation in vector-borne diseases. Infect Genet Evol 8:504–19.

Levin AM, de Vries RP, Conesa A, de Bekker C, Talon M, Menke HH, van Peij NNME, Wösten HAB. 2007. Spatial differentiation in the vegetative mycelium of *Aspergillus niger*. Eukaryot Cell 6:2311–22.

Libersat F, Delago A, Gal R. 2009. Manipulation of host behavior by parasitic insects and insect parasites. Annu Rev Entomol 54:189–207.

Libersat F, Gal R. 2013. What can parasitoid wasps teach us about decision-making in insects? J Exp Biol 216:47–55.

Linden H, Ballario P, Macino G. 1997. Blue light regulation in *Neurospora crassa*. Fungal Genet Biol 22:141–50.

Lone SR, Ilangovan V, Murugan M, Sharma VK. 2010. Circadian resonance in the development of two sympatric species of *Camponotus* ants. J Insect Physiol 56:1611–6.

Malek EA. 1980. Snail-transmitted parasitic diseases, Vol. I and II. Boca Raton (FL): CRC Press, Inc.

Manga-González MY, González-Lanza C, Cabanas E, Campo R. 2001. Contributions to and review of dicrocoeliosis, with special reference to the intermediate hosts of *Dicrocoelium dendriticum*. Parasitology 123:91–114.

Mitra R, Sapolsky RM, Vyas A. 2013. *Toxoplasma gondii* infection induces dendritic retraction in basolateral amygdala accompanied by reduced corticosterone secretion. Dis Model Mech 6:516–20.

Moore J. 2002. Parasites and the behavior of animals. New York: Oxford University Press.

Nobile CJ, Mitchell AP. 2007. Microbial biofilms: e pluribus unum. Curr Biol 17:R349–53.

O'Donnell AJ, Mideo N, Reece SE. 2013. Disrupting rhythms in *Plasmodium chabaudi*: costs accrue quickly and independently of how infections are initiated. Malar J 12:9.

O'Donnell AJ, Schneider P, McWatters HG, Reece SE. 2011. Fitness costs of disrupting circadian rhythms in malaria parasites. Proc R Soc B 278:2429–36.

Pontoppidan M-B, Himaman W, Hywel-Jones NL, Boomsma JJ, Hughes DP. 2009. Graveyards on the move: the spatio-temporal distribution of dead *Ophiocordyceps*-infected ants. PLoS One 4:e4835.

Poulin R. 1995. ''Adaptive'' changes in the behaviour of parasitized animals: a critical review. Int J Parasitol 25:1371–83.

Poulin R. 2010. Parasite manipulation of host behavior: an update and frequently asked questions. In: Brockmann H, Roper TJ, Naguib M, Wynne-Edwards KE, Mitani JC, Simmons LW, editors. Advances in the study of behavior, Vol. 41. Burlington: Elsevier, Inc. p. 151–86.

Prandovszky E, Gaskell E, Martin H, Dubey JP, Webster JP, McConkey GA. 2011. The neurotropic parasite *Toxoplasma gondii* increases dopamine metabolism. PLoS One 6:e23866.

Richter K, Kauser G, Bidmon HJ. 1989. Interaction of ecdysteroids with the nervous system. In: Koolman J, Verlag GJ, editors. Ecdysone. Stuttgart/New York. pp. 319–26.

Roenneberg T, Kuehnle T, Pramstaller PP, Ricken J, Havel M, Guth A, Merrow M. 2004. A marker for the end of adolescence. Curr Biol 14:R1038–9.

Roenneberg T, Merrow M. 2003. The network of time: understanding the molecular circadian system. Curr Biol 13:R198–207.

Sanjuán T, Guillermo Henao L, Amat G. 2001. Spatial distribution of *Cordyceps* spp. (Ascomycotina: Clavicipitaceae) and its impact on the ants in forests of Amazonian Colombian foothill. Rev Biol Trop 49:945–55.

Schmid-Hempel P. 1998. Parasites in social insects. Princeton (NJ): Princeton University Press.

Sharma VK. 2003. On the significance of circadian clocks for insects. J Indian Inst Sci 83:23.

Sharma VK, Lone SR, Goel A, Chandrashekaran MK. 2004. Circadian consequences of social organization in the ant species *Camponotus compressus*. Naturwissenschaften 91:386–90.

Spindler EM, Zahler M, Loos-Frank B. 1986. Behavioural aspects of ants as second intermediate hosts of *Dicrocoelium dendriticum*. Z Parasitenkd 72: 689–92.

Tenter AM, Heckeroth AR, Weiss LM. 2000. *Toxoplasma gondii*: from animals to humans. Int J Parasitol 30:1217–58.

Thomas F, Adamo S, Moore J. 2005. Parasitic manipulation: where are we and where should we go? Behav Process 68:185–99.

Thomas F, Poulin R, Brodeur J. 2010. Host manipulation by parasites: a multidimensional phenomenon. Oikos 119:7.

Thompson SN, Kavaliers M. 1994. Physiological bases for parasite-induced alterations of host behavior. Parasitology 109:119–38.

Vafopoulou X, Steel CGH. 1998. A photosensitive circadian oscillator in an insect endocrine gland: photic induction of rhythmic steroidogenesis *in vitro*. J Comp Physiol A 182:343–9.

Veening JW, Igoshin OA, Eijlander RT, Nijland R, Hamoen LW, Kuipers OP. 2008a. Transient heterogeneity in extracellular protease production by *Bacillus subtilis*. Mol Syst Biol 4:184.

Veening JW, Smits WK, Kuipers OP. 2008b. Bistability, epigenetics, and bet-hedging in bacteria. Annu Rev Microbiol 62:193–210.

Vinck A, de Bekker C, Ossin A, Ohm RA, de Vries RP, Wösten HAB. 2011. Heterogenic expression of genes encoding secreted proteins at the periphery of *Aspergillus niger* colonies. Environ Microbiol 13:216–25.

Vyas A. 2013. Parasite-augmented mate choice and reduction in innate fear in rats infected by *Toxoplasma gondii*. J Exp Biol 216:120–6.

Vyas A, Kim SK, Giacomini N, Boothroyd JC, Sapolsky RM. 2007. Behavioral changes induced by *Toxoplasma* infection of rodents are highly specific to aversion of cat odors. Proc Natl Acad Sci USA 104:6442–7.

Wagner-Smith K, Kay SA. 2000. Circadian rhythm genetics: from flies to mice to humans. Nat Genet 26:23–7.

Wang W, Barnaby JY, Tada Y, Li H, Tör M, Caldelari D, Lee DU, Fu XD, Dong X. 2011. Timing of plant immune responses by a central circadian regulator. Nature 470:110–4.