

Parasite-mediated protection against osmotic stress for *Paramecium caudatum* infected by *Holospira undulata* is host genotype specific

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Introduction

Interactions between different organisms, such as parasites and hosts, are influenced by their environment (Lazzaro & Little, 2009; Wolinska & King, 2009). In general, the more stressful the environment, the more costly it is for a host to harbour parasites (Bedhomme *et al.*, 2004; Restif & Kaltz, 2006; Tseng, 2006). Thus, under more stressful conditions, selection for resistant hosts should become stronger.

However, some alleged parasites have been found to aid their hosts in stressful environments (Michalakis *et al.*, 1992; Brownlie & Johnson, 2009). Such conditional benefits to infection by otherwise costly symbionts are crucial for the evolution of normally parasitic relationships towards mutualism (Fellous & Salvaudon, 2009), a situation in which both partners benefit from the interaction (Bronstein, 1994).

Still little is known about the factors and conditions that affect the beneficial effects of parasitism, in particular regarding the generality of such observations. For example, in various systems, the sign and magnitude of antagonistic

Abstract

Under certain conditions, otherwise parasitic organisms may become beneficial to their host. Parasite-mediated heat and osmotic stress resistance have been demonstrated for *Paramecium caudatum*, infected by several species of parasitic bacteria of the genus *Holospira*. Here, using the micronucleus-specific bacterium *Holospira undulata*, we investigate how infection mediates the response of two genotypes (clones 'K8' and 'VEN') of *P. caudatum* to heat (35 °C) and osmotic (0.24% NaCl) stress. In contrast to previous findings, we find no evidence for heat stress protection in infected individuals. We do, however, show an effect of symbiont-mediated osmotic stress resistance for the K8 clone, with infected individuals having higher survival than their uninfected counterparts up to 24 h after the onset of salt exposure. Despite this, both infected and uninfected individuals of the VEN clone showed higher survival rates than clone K8 individuals under osmotic stress. Thus, it would seem that parasite-mediated stress protection is restricted to certain combinations of host genotypes and types of stress and does not represent a general phenomenon in this system.

interactions have been shown to depend on complex interactions between environmental conditions and the genetic identity of the host and the parasite (Lambrechts *et al.*, 2006; Tétard-Jones *et al.*, 2007; Wolinska & King, 2009). It is thus conceivable that certain genotypes are protected by symbionts against environmental stress, while others are not (Salvaudon *et al.*, 2008). Moreover, organisms often face various types of stress and it is unclear whether the same symbiont can protect against multiple stresses, in particular, when they act simultaneously, and whether such a response is influenced by the host (or parasite) genotype.

We investigated the potential for multiple stress protection and the role of host genotype in the association between the ciliate *Paramecium caudatum* and its bacterial symbiont, *Holospira undulata*. Organisms that inhabit freshwater face buffered, but sometimes challenging changes in their environment. Temperature and ion concentration, among other things, can vary and threaten the survival of maladapted species and genotypes. Indeed, species of the genus *Paramecium* are well known to be highly sensitive to temperature

or salinity stress (Wichterman, 1986). However, under these conditions, survival of *P. caudatum* can be enhanced when harbouring bacterial symbionts of the genus *Holospira* (Smurov & Fokin, 1998; Hori & Fujishima, 2003; Fujishima *et al.*, 2005; Hori *et al.*, 2008). Under standard conditions, these symbionts are normally considered parasites that reduce host division and survival (Kaltz & Koella, 2003; Fokin, 2004; Restif & Kaltz, 2006; Nidelet *et al.*, 2009). However, at an elevated temperature (35 °C), infection with *Holospira obtusa* or *Holospira elegans* increases host survival (Hori & Fujishima, 2003; Hori *et al.*, 2008). Similarly, *H. undulata* and *H. obtusa* can increase survival under osmotic stress (Smurov & Fokin, 1998). These positive effects may be explained by the constitutive overexpression of hsp70 heat-shock proteins (hsps) in infected individuals (Hori & Fujishima, 2003). Heat-shock proteins are involved in general physiological responses to stress (Feder & Hofmann, 2003). As chaperones, they stabilize the conformation of proteins and ensure the resistance of the cell's biochemical pathways to environmental perturbations. The elevated expression of hsp70 in *Holospira*-infected paramecia may be a host response to reduce physiological disturbance caused by infection. The already elevated expression of heat-shock proteins then doubles up as protection against other types of stresses. It is, though, questionable whether a host genotype already resistant to a particular environmental challenge would benefit from symbiont-mediated protection. We therefore may expect host genotypes that vary in their tolerance to stressful environments to benefit differently from infection.

Here, we investigate whether *H. undulata* confers increased tolerance to two laboratory strains of *P. caudatum* to heat stress, and investigate whether there is extended protection against osmotic stress. We identify (1) how host survival differs between the two clones under osmotic and heat stress; (2) how the combined effects of these abiotic stresses affect host survival; and (3) how infection modifies the effects of these stresses on host life history.

Material and methods

Study organisms

Paramecium caudatum is a freshwater ciliate found in still water bodies in the northern hemisphere that feeds on bacteria and detritus within the water column (Wichterman, 1986). Reproduction is predominantly asexual through mitotic division, with most gene expression occurring in the polyploid macronucleus; sexual reproduction occurs by conjugation between different mating types (E and O). Under exponential growth conditions, *P. caudatum* divides up to three times every 24 h. Optimal growth temperatures generally range between 24 and 28 °C, depending on the species

(Wichterman, 1986). Similarly, species differ in their capacities to tolerate osmotic stress, allowing them to live in habitats of varying levels of salinity (e.g. brackish water; Smurov & Fokin, 1999). There is also evidence of within-species genetic variation in growth and survival at different temperatures (Fels & Kaltz, 2006). In our laboratory, cultures of *P. caudatum* are maintained at 23 °C, in a culture medium prepared from dried organic lettuce (Nidelet & Kaltz, 2007) and supplemented with the bacterium *Serratia marcescens* (strain A173, Institut Pasteur, Paris, France) for food.

Holospira undulata, a gram-negative alphaproteobacterium (Amann *et al.*, 1991), is a natural parasite of *P. caudatum*. Infection occurs through ingestion of infectious forms (15–20 µm) while feeding. Infectious forms are transferred to the micronucleus upon fusion with the parasite-containing food vesicle minutes after ingestion. After ~24 h, infectious forms differentiate into reproductive forms (5 µm), which multiply and begin to fill the micronucleus. After 7–10 days, an accumulation of reproductive forms will stimulate the production of infectious forms. Hosts can simultaneously harbour both spore types and a heavily infected host can be infected with up to several hundred infectious spores. Vertical transmission is achieved through segregation of reproductive forms into the two daughter micronuclei during asexual reproduction. Horizontal transmission occurs when infectious forms are released into the environment during host division or upon host death.

Host and parasite origins

Two host clones were used in this experiment. Clone K8 was established in 2002, by crossing strains KNZ 5 (mating type O3) and KNZ 2 (mating type E3; both strains provided by T. Watanabe, Tohoku University, Japan); the clone VEN (unknown mating type, originally collected near Venice, Italy) was provided by P. Flügel (Krakow, Poland) in 2007. In June 2009, we established mass cultures of each clone from single individuals. In order to investigate the effect of infection, we infected half of each mass culture with *H. undulata* 5 months before the beginning of our experiment. The parasite inocula comprised a mix of bacteria retrieved from six different infected *P. caudatum* clones; controls received a mock inoculum without bacteria. Mass cultures were distributed across 50-mL Falcon tubes.

Two subclones were created from each of the four cultures (2 clones × 2 infection status) to control for environmental variation between mass cultures. Each subclone originated from individual *Paramecium* cells chosen at random from the mass cultures and propagated for seven to eight generations before the onset of the experiment. Single *Paramecium* cells for the survival experiment came from each of these eight subclones (2 clones × 2 infection status × 2 founding *Paramecium*).

Following the protocol of Barth *et al.* (2006), we sequenced a fragment of the cytochrome oxidase subunit 1 (COI, on the mitochondrial genome) *P. caudatum* gene. We sequenced two independent subclones of each clone to be sure that both are genetically homogeneous. The total fragment length was > 800 bp, from which we selected a 300-bp region with the best sequence quality. Across this region, we identified 20 single-nucleotide polymorphisms that differed between clones (6.7% genetic distance between clones). We found no difference between subclones of the same clone. Sequencing the COI gene thus revealed the two clones to be genetically distinct at the sequence level in addition to their phenotypic differences.

Experimental protocol

We tested the effects of osmotic and heat stress, alone and in combination, on infected and uninfected *Paramecium*. Heat stress was delivered by placing the *Paramecium* in an incubator set to 35 °C. Osmotic stress was achieved by adding 0.24% NaCl (Sigma, France) to the salad medium. Salinity levels and temperatures are within the range of the ones for which *Holospora* has been shown to protect its host.

The effect of these conditions was determined on a single *Paramecium*. For each combination of temperature, salinity, infection and genotype, we phenotyped 45 individuals. These 45 cells were spread over five blocks, each containing nine individuals from each treatment. The individuals from the two subclones of each genotype–infection combination were separate among blocks: one subclone seeded three blocks and the other seeded the two remaining blocks. These blocks were used as the basic unit of replication in our statistical analyses.

Individual *Paramecium* were placed in 60- μ L drops of culture medium (with and without NaCl) arranged inside the lids of 24-microwell plates (NuncTM, Fisher Scientific, France). Each experimental block contained six plates comprising 144 *Paramecium*. Three of these plates were placed in an incubator at 23 °C and three at 35 °C. *Paramecium* clones, infected and uninfected individuals and salt exposure were arranged systematically across plates. Plates were sealed using ParafilmTM (Dutscher, France) and placed in a box containing a wet towel to minimize evaporation during the experiment. The paramecia were checked for survival and/or division 3, 6, 9 and 24 h after the start of the experiment.

Statistical analysis

We considered the ‘subclone’ as the relevant unit of replication for statistical analysis. Thus, for each experimental block, we calculated the proportion of survivors among the nine individuals per subclone and treatment. This yielded a total of 80 observations of proportion survival per time

point (2 clones \times 2 infection status \times 2 salt treatments \times 2 temperature treatments \times 5 blocks). As there was no significant effect of experimental block on proportion survival at any of the four time points (all $F_{4,75} < 2.09$, $P > 0.09$), this factor was excluded from all further analyses. We used factorial ANOVA to analyse, first, how temperature and osmotic stress affected the survival of uninfected *Paramecium* from each clone. Subclone was nested within host clone and crossed with salt and temperature treatments. Proportion survival at the different time points was arcsine-square-root transformed to meet assumptions of ANOVA. Where appropriate, initially, fully factorial statistical models were simplified by backward elimination of nonsignificant terms.

Second, we analysed the effects of the infection on the proportion survival in the presence or absence of abiotic stresses. Because of the high mortality under simultaneous salt and temperature stress, we did not have sufficient statistical resolution to analyse all four effects (infection status, temperature treatment, salt treatment and host clone) in a single ANOVA. Instead, we performed two separate analyses; one focussing on the temperature treatment and one on the salt treatment. Factorial statistical models were constructed by the same method as above, with the clonal replicate population nested within the host clone and infection status. All statistics were performed using the statistical software JMP version 5.0.1.2.

Results

Effects of abiotic stress (uninfected individuals only)

Both the high-salt and the high-temperature treatment reduced the survival of uninfected paramecia, but to different degrees (Fig. 1a and b). Overall, a 24-h exposure to 35 °C (in the absence of salt) reduced survival by *c.* 20%, relative to the 23 °C control treatment ($F_{1,14} = 6.59$, $P = 0.0223$). This reduction did not differ significantly between clones (temperature treatment \times clone interaction: $F_{1,14} = 0.83$, $P = 0.3765$).

The high-salt treatment strongly reduced survival, and > 50% of the paramecia died within the first 6 h of the experiment. Statistical analysis of survival after 6 h revealed a significant temperature treatment \times salt treatment \times clone interaction ($F_{1,30} = 6.22$, $P = 0.0184$). This indicates, first, a synergistic effect of the two stresses: simultaneous exposure to high-salt and high-temperature treatments produced disproportionate rates of mortality (Fig. 1a and b). More than 90% of the individuals died within 3 h; after 6 h, all paramecia were dead under combined stress. Second, one of the two clones (VEN) was more tolerant to salt stress, at

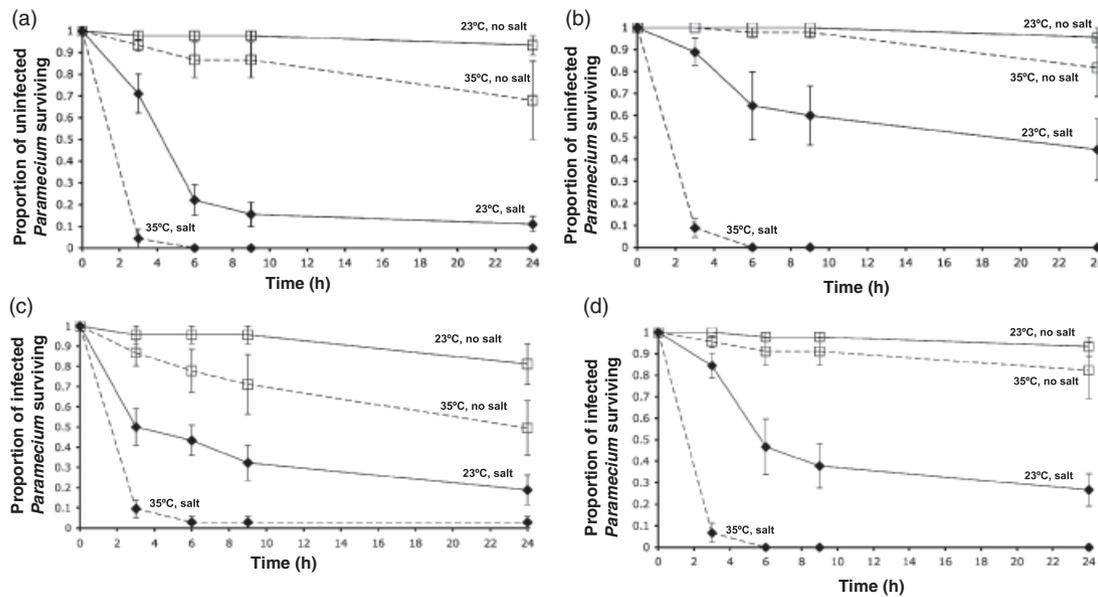


Fig. 1. Proportion of *Paramecium caudatum* surviving 3, 6, 9 and 24 h postexposure to osmotic and heat stress. (a) Survival for uninfected clone K8; (b) survival for uninfected clone VEN; (c) survival for infected clone K8; and (d) survival for infected clone VEN.

least at 23 °C, where this clone had a threefold higher survival than the other clone (K8; Fig. 1a and b).

Effects of infection

Temperature treatment

In the absence of salt, we did not observe a significant overall effect of infection on host survival after 24 h ($F_{1,4}=0.15$, $P=0.7169$, Fig. 2a and b), nor did the effect of infection vary significantly with temperature or host genotype (all interactions including infection status: $F < 0.2$, $P > 0.7$). Note that the first divisions occurred between 9 and 24 h. Overall, the proportion of paramecia that divided was more than twice as high for uninfected individuals ($51 \pm 6\%$) than for infected ones ($22 \pm 5\%$), but, again, the effect of infection did not significantly vary with temperature or host clone identity (both interactions: $P > 0.78$, in analysis on log-transformed number of individuals). Thus, there was no evidence for parasite-mediated heat-stress protection. At a high temperature, infection neither specifically increased nor decreased host survival and reproduction during the 24 h of the experiment.

Salt treatment

At 23 °C, significant salt treatment \times infection \times clone interactions for survival were detected as early as 6 h after the start of the experiment (Table 1). That is, the response to the salt treatment depended on both the infection status and the

clone identity. For the VEN clone, infection decreased survival in the high-salt treatment at 6, 9 and 24 h post-exposure; in contrast, for the K8 clone, infection increased survival in the high-salt treatment (see multiple contrasts; Fig. 3a and b). Thus, under osmotic stress, infection was costly for the VEN clone, while it was beneficial for the K8 clone. Despite this beneficial effect, both infected and uninfected VEN individuals had a higher general salt tolerance and thus higher survival than their K8 counterparts (Fig. 3a and b).

Discussion

The two *P. caudatum* genotypes used in this experiment differed in their response to osmotic and heat stress, as well as in the way this response was modified by infection with *H. undulata*. We first discuss the combined effects of the two abiotic stressors in the absence of infection; second, we focus on the interactions between stress, infection and host genotype.

Combination of biotic stresses in the absence of infection

Our experiment confirms the negative effects of heat and osmotic stress on the survival of uninfected *P. caudatum* (Wichterman, 1986; Smurov & Fokin, 1999; Fujishima *et al.*, 2005). Osmotic stress was more harmful than temperature stress, causing a 50% reduction in survival. The combined effects of the two stresses had a disproportionate effect on host survival, causing 100% mortality within 6 h. While the

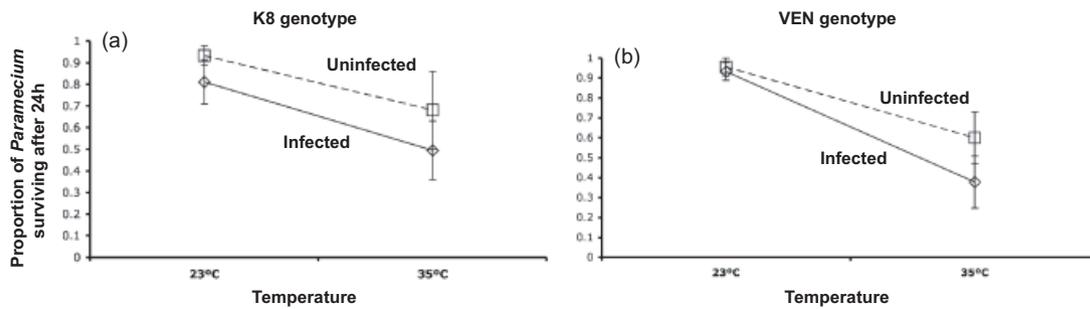


Fig. 2. Proportion of *Paramecium caudatum* surviving 24 h postexposure to heat stress: (a) clone K8 and (b) clone VEN.

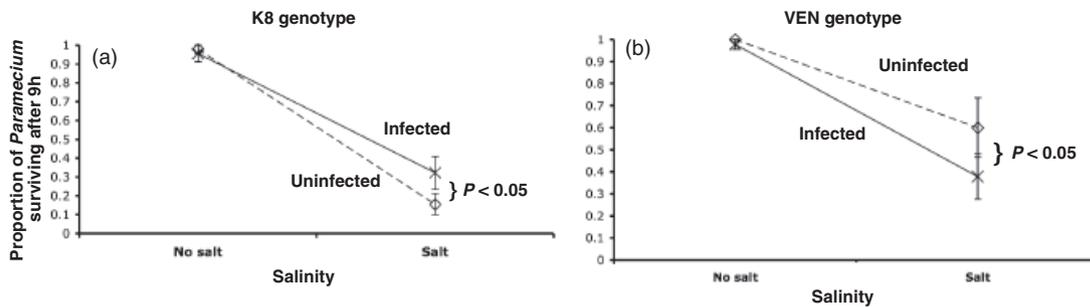


Fig. 3. Proportion of uninfected and infected *Paramecium caudatum* surviving 9 h postexposure to osmotic stress: (a) clone K8 and (b) clone VEN.

Table 1. Test statistics of the three-way interaction between infection status \times salt exposure \times host clone in ANOVA on the proportion of *Paramecium* surviving 3, 6, 9 and 24 h after exposure

	3 h	6 h	9 h	24 h
$F_{1,4}$	0.29	10.76	44.58	9.72
P -value	0.6162	0.0305	0.0026	0.0356

This interaction term (1 df) was tested over the corresponding interaction at the within-clone level [clonal replicate population \times salt exposure (infection status, salt exposure), 4 df].

two clones did not differ in their tolerance to heat stress, the VEN clone had a moderately higher tolerance to osmotic stress at 23 °C. These results illustrate that it can be difficult for an organism to simultaneously manage two environmental stresses.

Tolerance to heat stress in many organisms including *Paramecium* involves the upregulation of heat-shock proteins. This tolerance mechanism against a number of environmental stresses is conserved across the animal kingdom (Feder & Hofmann, 2003). It is unknown whether they function for tolerance against osmotic stress in *P. caudatum*; however, their upregulation has been observed in other ciliate species adapted to osmotic conditions (Plekhanov *et al.*, 2006). Osmoregulation in *Paramecium* relies on feedback between the internal mechanisms that control the osmolarity of the cell and permeability of the plasma

membrane. *Paramecium* adapted to osmotic environments equal to or higher than, their intracellular environment will increase the ion concentration in their cells so that they can continue normal activity (Stock *et al.*, 2002). However, there must be a limit beyond which they can no longer increase this concentration when water is drawn from the cell, causing it to rupture. Our results show that *Paramecium* cannot tolerate two abiotic stresses simultaneously. Whether or not heat-shock proteins assist in tolerance to osmotic stress remains unclear. However, the high mortality under combined heat and osmotic stresses suggests that some of the tolerance mechanisms against these two stresses differ. A conflict may therefore arise between their simultaneous functioning, leading to a failure of either to function properly.

Genetic variation between the two clones for tolerance against osmotic stress suggests that selection can act on this trait and that clone VEN would have a selective advantage over clone K8 under high-salt conditions. This difference in salt tolerance could be attributable to a difference in the origin of the two clones and a difference in their evolutionary histories in environments with different salinities. Differences in salt tolerance are known between closely related *Paramecium* species, probably attributable to their origin and to salinities they are adapted to (Smurov & Fokin, 1999). However, one should note that our two clones have spent several years in the lab before we conducted this assay.

It is therefore difficult to attribute phenotypic differences to the evolutionary origins of each clone.

Effect of infection

Previous studies revealed that *Paramecium* infected with *Holospira* spp. have increased tolerance to heat and osmotic stress (Smurov & Fokin, 1998; Hori & Fujishima, 2003; Fokin, 2004; Fujishima *et al.*, 2005; Hori *et al.*, 2008). In this experiment, we found that infection provided protection against osmotic stress only, and this was true for only one host genotype.

Contrary to reports with other *Holospira* species in other *Paramecium* genotypes, infection did not protect the host against heat stress. Nor did we find a synergistic effect of temperature and infection where the parasite becomes more virulent in more stressful environments, as observed frequently in other systems (e.g. Bedhomme *et al.*, 2004; Jokela *et al.*, 2005). Infected individuals were observed to have lower levels of division, although this was not affected by temperature. The absence of an effect of infection on survival at different temperatures may be due to the host genotypes used in this experiment. Differences in the life-history traits for infected and uninfected *Paramecium* in various environments are well-documented (Kaltz & Koella, 2003; Fels & Kaltz, 2006; Restif & Kaltz, 2006), and it would seem that the two genotypes used in this experiment respond similarly to heat stress, infected and uninfected. Alternatively, it could also be that *H. undulata* does not provide protection against heat as, to our knowledge, such an effect has never been described before.

There was a difference between the two clones in their response to osmotic stress. Consistent with previous findings, we find evidence for parasite-mediated protection for clone K8, infected individuals showing higher survival. The opposite was true for clone VEN, with infected individuals showing lower survival throughout the experiment, including under osmotic stress. One must be cautious in generalizing to species the observations based on one clone. Our results do not question the validity of the repeated observation that a *Holospira* symbiont can protect paramecia against abiotic stresses, but they do suggest that these effects may not be as universal as previously thought.

Differences between genotypes in their tolerance to abiotic stress when infected may arise through parasite-induced upregulation of heat-shock proteins (Hori & Fujishima, 2003). There is then no reason why an already tolerant genotype should benefit from infection, being able to upregulate heat-shock proteins itself or already having high constitutive levels of hsp's. This may be true for the VEN clone in environments presenting an osmotic stress. Nonetheless, all infected paramecia must pay a cost to carrying the symbiont and providing the resources necessary

for its growth. This cost may be offset, and thus hidden, when the host benefits from symbiont-induced tolerance to stress, but is visible when the host does not need the symbiont. This cost may be reflected by the lower survival we observe for infected VEN individuals.

The observation that different host genotypes do not benefit equally from infection relates to the standing genetic variation for tolerance against osmotic stress and the mechanisms by which stress adaptation may occur. This observation has broad consequences for the evolution of host–parasite interactions towards mutualistic relationships. If a host already possesses tolerance to an abiotic stress, symbiont-mediated protection should provide no benefit. Consequently, an already tolerant genotype, such as for clone VEN, should not gain from infection and instead pays the cost of infection, as we observe. Conversely, when a genotype benefits from symbiont-mediated protection in stressful environments, selection should favour infected individuals and the symbiont may become fixed in the population, possibly leading to mutualism. We need to be cautious, though, when interpreting these results. Selection would favour infected K8 hosts in a monoclonal population facing osmotic stress. However, this population would be easily invaded by both infected and uninfected VEN hosts, both having higher levels of survival than infected K8 hosts under osmotic stress. Our results support recent theory for the evolution of mutualism when symbionts help their hosts (Fellous & Salvaudon, 2009), and underline the complex interactions present in natural systems between hosts and parasites.

Conclusions and perspectives

This experiment reveals how different host genotypes respond to combinations of biotic and abiotic stresses. The lack of a general finding for parasite-mediated protection against abiotic stress for both genotypes highlights the need for multiple genotypes to be included in future investigation. Further, we show how infection with a different, but closely related parasite, can differentially affect host life history. We cannot be sure whether this result is attributable to host genotype or parasite species. It would be interesting to extend this work to see how different parasites affect the host when faced with different abiotic stresses and to observe the direct effects of abiotic stress on parasite life history. In particular, the survival of parasite spores outside the host when faced with thermal and osmotic stress and also whether prior exposure of an infected host to such environments confers a selective advantage for the parasite. Such findings would not be surprising and would highlight the complex dynamics present in host–parasite interactions. Finally, our results reflect the survival of single *Paramecium* cells. The costs and benefit to infection that we measured

may not be directly extrapolated to populations as the effects of parasites may change depending on the population structure (Bedhomme *et al.*, 2005). Further work is thus required to understand how combinations of infection and abiotic stresses interact with the competition present in *Paramecium* microcosm populations.

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Authors' contribution

A.B.D. and S.F. contributed equally to this work.

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