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Synergistic effects of dual parasitism in *Daphnia magna* under nutrient limitation

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ABSTRACT. Human-induced increases in the bioavailability of carbon (C), nitrogen (N) and phosphorus (P) have the potential to alter the context for host-parasite dynamics in aquatic ecosystems. Given that both eutrophication and infectious diseases are becoming more prominent, it is essential to disentangle the factors that determine virulence expression in keystone grazers. Here, we focus on the impact of nutrient limitation in single *versus* dual parasite exposure in the water flea *Daphnia magna* (Crustacea, Branchiopoda). For this, we fed specimens of *D. magna* with algae differing in C:N:P ratios and exposed them to two virulent parasites, *Pasteuria ramosa* (bacteria) and the agent causing White Fat Cell (WFCD, unknown classification), both in single and dual parasite exposure treatments. Exposure to the two parasites synergistically reduced host survival, mainly driven by WFCD exposure, especially under severe nutrient limitation. Under these conditions individuals of *D. magna* began reproducing earlier, which resulted in a higher reproductive output upon dual parasite exposure. We here discuss these results within the framework of host stress responses, nutrient allocation and energy budgets, and conclude that the way food quality interferes with host-parasite interactions varies, depending on the parasite species involved, the nutrient requirements of all actors and the trait investigated.

KEYWORDS. Energy allocation, food quality, host-parasite, synergism.

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Introduction

Natural populations in food webs are strongly regulated by consumers such as parasites, but also bottom-up by food abundance and quality (LAFFERTY *et al.* 2008; PRESTON & JOHNSON 2010; AALTO *et al.* 2015). Host and parasite traits are sensitive to environmental factors such as food availability and quality, which interact to affect parasite virulence levels (FELLOUS & KOELLA 2009; VALE *et al.* 2013; DUNCAN *et al.* 2014). The outcome of the interaction between host and parasite depends on the amount of energy available for the host, the allocation of this energy towards life history responses, and the need and capacity of the parasite to exploit this (SMITH & HOLT 1996; BEDHOMME *et al.* 2004; PEDERSON & FENTON 2007; MIDEO 2009; CRESSLER *et al.* 2014). In general, parasite virulence is expected to increase upon nutrient limitation of the host, due to increased resource competition between both antagonists (FERGUSON & READ 2002; LAMBRECHTS *et al.* 2006; FROST *et al.* 2008; CORNET *et al.* 2014). Resource

availability will also affect (especially costly) host immune defenses (OJALA *et al.* 2005; KAPARI *et al.* 2006; KLEMOLA *et al.* 2007; LITTLE & KILLICK 2007), especially if there is a metabolic link between their resources, given that host immunity and parasites then interact as “competitors” (CRESSLER *et al.* 2014). On the other hand, a parasite that is not efficient in stealing nutrients from its host may also show a reduced growth rate, which is then reflected in lower virulence levels (EBERT *et al.* 2004; SEPPÄLA *et al.* 2008; ABU KWAİK & BUMANN 2013; CORNET *et al.* 2014).

In most cases, multiple infections will be more virulent than single infections, given that the most competitive strains gain a disproportional share of the host (BEN-AMI *et al.* 2011; SCHMID-HEMPEL 2011; ALIZON *et al.* 2013; GRIFFITHS *et al.* 2015). However, the net outcome of multiple infections depends, inter alia, on the mode of competition between different parasite species, the involvement of the host immune system (GRAHAM 2008; MIDEO 2009; CRESSLER *et al.* 2014), host demography (IZHAR *et al.* 2015) and the environmental dependency of multiple infections (PENCZYKOWSKI *et al.* 2016). In multiple infections, the combined negative effect of the parasites will depend on whether the parasites require nutrients essential to the host. Also, the type of interaction between the parasites will be important: antagonistic interactions between parasites can decrease virulence, while a reciprocal facilitation could synergistically lead to increased virulence levels (ESWARAPPA *et al.* 2012; AALTO *et al.* 2015).

In freshwater aquatic ecosystems, biochemical and elemental composition of natural seston fluctuates considerably and affects food web dynamics (SØNDERGAARD *et al.* 1999; DE SENERPONT DOMIS *et al.* 2013), for instance via the microbial community (ELSER *et al.* 1995; COTNER *et al.* 2010; PONCE-SOTO *et al.* 2015), but also via zooplankton performance in terms of reproduction, mortality, growth rate and community composition (STERNER *et al.* 1993; VERREYDT *et al.* 2012; SARPE *et al.* 2014). For instance, the keystone grazer *Daphnia* (Crustacea, Branchiopoda) requires a sufficient amount of P to invest in P-rich ribosomal RNA (rRNA), which is needed for protein synthesis and growth (ELSER *et al.* 2000, 2003). Nutrient deprivation will also affect trade-offs between life history traits, e.g., prolonging life span, but at the cost of reproduction (BOGGS 2009; PIETRZAK *et al.* 2010).

A shortage of energy and elements impacts the interaction of *Daphnia* with its parasites (FROST *et al.* 2008; HALL *et al.* 2009; DALLAS & DRAKE 2014; AALTO *et al.* 2015; DECAESTECKER *et al.* 2015). In natural systems, parasitism of *Daphnia* individuals is the rule rather than the exception, with multiple infections occurring frequently (DECAESTECKER *et al.* 2005; EBERT 2005; WOLINSKA *et al.* 2009; JANSEN *et al.* 2010; LANGE *et al.* 2014). Because of their tight association, parasites can compete for nutrients with their host (FROST *et al.* 2008; HALL *et al.* 2009). We here investigate how severe food deprivation impacts the virulence of single *versus* multiple parasite exposure in *Daphnia magna* (Straus, 1820). More in particular, we performed a laboratory experiment in which we infected individuals of *Daphnia magna* with the bacterium *Pasteuria ramosa* and the infectious agent that causes White Fat Cell Disease (EBERT 2005; COOPMAN 2014), both in a single species and multiple species infection. These infected hosts were then fed with food of different C:N:P ratios, in order to monitor how changing food quality impacts host-parasite interactions. We focussed on algal C:N:P ratio as this is known to induce P-limitation in *Daphnia* (ACHARYA *et al.* 2004), but the tested effect can also be impacted by food quality in general, as a high N:P ratio correlates with other food parameters such as digestibility and PUFA (poly-unsaturated fatty acids) content.

Material and methods

Daphnia genotypes and parasites

The *Daphnia magna* genotypes in our experiment (clones OM2 10.20, 12.1, 22.11, 25.10, 25.6) were from a shallow pond (Oude Meren 2, ‘Abdij van ‘t Park’, Heverlee, Belgium, 50°51' N, 04°43' E). The parasites used in this experiment were *Pasteuria ramosa* and the agent causing White Fat Cell Disease (WFCF). *P. ramosa* is a horizontally transmitted parasite infecting the hemolymph, causing chronic

TABLE 1

Elemental composition of the algal (*Scenedesmus obliquus*) cultures.

Culture	Elemental concentration (mg/L)			Elemental ratio (molar)		
	N	P	C	N:P	C:N	C:P
1	1.56	0.137	59.22	26	22	575
2	6.07	0.121	103.86	114	9.75	1136

infections, resulting in a sterilized *Daphnia* sp. host individual (EBERT 2005). Feeding individuals of *Daphnia* sp. with P-deprived food induces increased virulence of *P. ramosa* (FROST *et al.* 2008). WFCD is a small coccoid parasite that infects the adipose tissue of *Daphnia* species (EBERT 2005). The infection is transmitted horizontally, and severely impacts survival in *Daphnia* species (COOPMAN *et al.* 2014; LANGE *et al.* 2014) and to a lesser extent fecundity upon disease progression. *P. ramosa* strains were isolated from the same pond as were the *Daphnia* clones, but from a later time (2012) than the clones were isolated (most recent clone isolates 2002). WFCD strains were isolated from a shallow pond in Zonhoven, Belgium (lake 21, 50°59'12.72" N and 5° 0'35.56" E) and have been cultured in OM2 clones during a field season (180 L outdoor mesocosms).

Algal elemental ratio in the experimental set-up

The green alga *Scenedesmus obliquus* (Kützing, 1833; Chlorophyta, Chlorophyceae) was grown in semi-continuous batch cultures (dilution rate 0.1d⁻¹). We used artificial WC-medium in which all nutrient concentrations were reduced tenfold to simulate mesotrophic conditions (except for NaNO₃ and K₂HPO₄·3H₂O). The P-concentration in the medium was kept constant [K₂HPO₄ = 0.2mg/L] and the N-concentration was raised (from ± 1.5 to ± 12mg/L with resulting molar N:P ratios of 16 and 128) to induce differences in C:N:P ratios. An N:P ratio of 128 in chemostats has previously been used to induce severe P-limitation in *Daphnia* (STERNER *et al.* 1993; ACHARYA *et al.* 2004). These food quality treatments were not chosen to mimic the natural environment of the *Daphnia* individuals, but rather to generate a gradient ranging from low to extreme food limitation. By manipulating the absolute N content rather than decreasing P in the medium, we aimed to induce severe food limitation in the *Daphnia* (rather than solely implementing P-limitation), as both a high absolute N-concentration and high N:P ratio have been shown to be detrimental for *Daphnia* (FROST *et al.* 2008; DALLAS & DRAKE 2014). For each *S. obliquus* culture, we measured the algal C, N and P-content. Aliquots of algal suspension were filtered over pre-ignited Whatman GF/F filters. These filters were kept dry at 60°C before analysis. P and N-concentrations of *S. obliquus* were determined via persulfate digestion followed by molybdate-ascorbic acid colorimetry using a microflow technicon™ segmented flow analysis system (Quattro, Seal Analytical). We determined C-content on the same filters by dividing algal dry weight (measured as in DECLERCK *et al.* 2007) by two (assumption of 50% carbon content of algal dry weight). The absolute concentrations and elemental ratios for C, N and P are given in Table 1. As the optimal elemental ratio for *Daphnia* ranges between 100–200 for C:P and 12–28 for N:P (ANDERSEN & HESSEN 1991; HESSEN & LYCHE 1991; ELSER *et al.* 2000; FROST *et al.* 2006; JEYASHINGH *et al.* 2009, but see DEMOTT & VAN DONK 2013), we can say that nutrient limitation was present in both algal cultures. However, nutrient limitation was stronger in algal culture 2, apparent from the high (but still realistic, STERNER 2008) C:P and N:P ratios clearly indicating strong P-limitation. However, although these algal ratios strongly suggest nutrient limitation, we cannot exclude the possibility that other food quality parameters such as PUFA content and digestibility might affect the *Daphnia*. Therefore, we now refer to the effect of “food quality / limitation / deprivation” rather than “nutrient limitation / deprivation”. To standardize the amount fed (based on C-concentration) to the individuals of *D. magna*, we measured the algal C-content spectrophotometrically (Hach Lange DR 2800) using a calibration curve for each *S. obliquus* culture. These curves were established by measuring the optical density at 750nm of the algal suspension with known C-concentration.

Experimental set-up

After hatching, the *D. magna* genotypes were kept as stock cultures under standardized conditions ($20^{\circ}\text{C} \pm 2^{\circ}\text{C}$, 16/8 h day / night cycle) and were fed *ad libitum* with *S. obliquus*. For each genotype (five genotypes in total), we grew five separate maternal lines. Each maternal line was kept for at least two generations in the laboratory before the start of the experiment. At the first day of the experiment, one-day old juveniles were transferred to 80ml jars, with a total of five juveniles per jar. Each jar contained one individual for each genotype. As it has been shown that there are strong genotype x genotype interactions with respect to *Daphnia* – *P. ramosa* interactions (CARIUS *et al.* 2001; DECAESTECKER *et al.* 2007, 2013; METZGER *et al.* 2016), we pooled five genotypes in one experimental jar to overcome such specific host-genotype – parasite strain interactions with respect to infectivity, given that the main interest of this study was on virulence effect. By pooling different genotypes, the chance of infection is higher and as such there is a higher chance of variation in the virulence effect. In total, 80 experimental jars were set up (four parasite treatments $\times$ two food quality treatments $\times$ ten replica per treatment combination). All experimental replicas contained the same combination of these five particular genotypes. Because of this, and because of the fact that it was impossible to identify a particular genotype on a visual basis, *Daphnia* genotype could not be included in the statistical analysis. From the first day of the experiment, all *D. magna* individuals were given a 2 mg C/L algal solution of the corresponding food ratio. All cultures were refreshed every other day, except when infections were initiated. At day four of the experiment, infections were initiated. For this, we transferred all *D. magna* individuals to a volume of 40 mL to maximize spore uptake. To obtain the spore suspensions for each parasite, cadavers of *D. magna* individuals (infected with WFCD or *P. ramosa*) were homogenized mechanically by using a pestle. This homogenized tissue, containing spores of the specific parasite species (WFCD or *P. ramosa*), was then added to their respective experimental jars to initiate the infections (WFCD, *P. ramosa* or the combined parasite treatment). To exclude food effects of adding the homogenized *Daphnia* tissue, the placebo (no parasite) treatment was initiated by exposing the *D. magna* individuals to homogenized cadavers of healthy, uninfected *Daphnia* individuals. Each of the experimental treatments received an equal amount of homogenized (infected or uninfected) *Daphnia* tissue.

Spore concentrations of *P. ramosa* in this homogenized tissue were estimated with a counting chamber (0.1 mm depth, Bürker counting chamber) using phase-contrast microscopy at 400 $\times$ magnification. For the *P. ramosa* and the combined parasite treatment, we chose an infection dose of 75 000 *P. ramosa* spores per *D. magna* individual to ensure a very high infection rate (REGOES *et al.* 2003). The causative agent of WFCD, presumably a virus (Ebert and Tönshoff, personal communication), can hardly be detected under a light microscope, which makes it impossible to determine spore loads of this parasite. Therefore, to initiate the WFCD and combined parasite treatment, we used one homogenized *D. magna* cadaver, infected with WFCD, to infect three experimental individuals. During the first three days of the parasite exposure, the amount of medium was kept low (gradually increasing from 20 mL to 40 mL). Jars were not refreshed for four days and were stirred daily to maximize spore uptake by *D. magna* individuals. After 33 days, *Daphnia* mortality in all experimental treatments was very high and the experiment was terminated. Survival and reproduction (time to first clutch and the total number of juveniles produced per jar) of *D. magna* individuals were monitored daily.

Prediction of joint effects of parasites

Interactions between *P. ramosa* and WFCD were tested by comparing predicted and observed effects of the combined treatment. Combined parasite effects were estimated based on the single parasite treatment effects according to Equation 1 (based on the model by BLISS 1939, see also COORS & DE MEESTER 2008).

$$E_{\text{mix}} = 1 - \Pi^i (1 - E_i) \quad E_{\text{mix}} = 1 - \Pi^i (1 - E_i) \quad \text{Eqn 1}$$

E_{mix} represents the estimated combined effect of the parasites, while E_i stands for the effect of the single stressor i . E_i represents a transformed value of the observed value e_i for the single stressor based on Equation 2.

$$E_i = \frac{(c_i - c_{\text{control}})}{(c_{\text{max}} - c_{\text{control}})} \quad E_i = \frac{(c_i - c_{\text{control}})}{(c_{\text{max}} - c_{\text{control}})} \quad \text{Eqn 2}$$

e_i is the value of the single stressor treatment, while e_{max} represents the value of the maximum possible effect for the single stressor. In the case of *D. magna* survival, this value was 0. For the number of juveniles produced, the maximum possible effect was defined based on the range of the data. The maximum number of juveniles produced here was set at $e_{\text{max}} = 15$. Parasites were considered to act as synergistically or antagonistically, when the predicted values were outside the range of $2 \times \text{SE}$ of the observed effect (based on COORS & DE MEESTER 2008).

Statistical analysis

In all analyses, the food-treatment effect was expressed as ‘limited’ versus ‘extremely limited’. Both the parasite (“P” in Table 2) and food limitation (“FQ” in Table 2) effect were considered as factorial variables. Survival was analyzed in two different ways. First we performed an overarching survival analysis using recordings of all time points (survival was monitored on each day of the experiment), using a Cox proportional hazards model with the `coxph()` specification in R. This analysis was performed to relate the time that passed before death to changes in food quality and parasitism, and to account for individuals that survived up until the end of the experiment. All explanatory variables were considered to be static (*i.e.* their impact did not change over time; DUNEAU *et al.* 2012; DUFFY *et al.* 2015). This analysis predicts the relative risk of death for each *D. magna* individual, which is inversely correlated with the linear predictor (LP) value used here for visualization of the data (thus the higher the LP value, the lower the risk of dying early in the experiment). We used these LP values rather than the relative risk as they most correctly represent the outcome of the survival analysis. As there were five individuals per jar, survival values for the *D. magna* individuals from the same jar were considered to be depending on the jar in which they were cultured. This jar dependency was integrated in our analysis by defining “jar” as a random factor. In a second analysis, we zoomed in on *D. magna* survival on day 14, as WFCD is known to exert strong virulence effects from this day and further (EBERT *et al.* 2005), and the final day (day 33) of the experiment. Survival on day 14 and day 33 was analysed using a repeated measures generalized linear model (rmGLM), as measures on experimental jars on day 14 and 33 were not independent (“T” in Table 2). In this rmGLM, survival (number of surviving individuals per jar) was considered to follow a binomial distribution. Additionally, we analysed the number of juveniles produced per experimental jar in a generalized linear model (GLM), with the number of juveniles assumed to follow a Poisson error distribution. However, the model for the number of juveniles produced showed a significant level of over-dispersion, so a quasi-Poisson distribution was used instead. For the time to release the first clutch we used a Cox proportional hazards model by using the `coxph()` specification in R. We chose this model instead of a traditional ANOVA as the Cox proportional hazards model includes individuals that did not reproduce at the end of the experiment (day 33). The outcome of this analysis generates a linear predictor, which relates to the “reproduction hazard”, *i.e.*, the chance to reproduce early in the experiment. As these values most correctly represent the outcome of the Cox survival analysis, we used these values for the visual representation of the data. For each model, we used the AIC criterium to select the best subset of parameters. Post-hoc comparisons between the parasite treatments were made for means within one food quality treatment (when the parasite $\times$ food quality interaction effect was significant) or for means averaged over the food quality treatments (when there was no parasite $\times$ food quality interaction but a significant main parasite effect). Post-hoc comparisons were made using the `glht {multcomp}` function with the `mcp = “Tukey”` specification in R.

Results

An increased degree of nutrient limitation significantly reduced *Daphnia* survival and reproduction (Fig. 1, Table 2). The overarching survival analysis revealed a significant effect of altered food quality on *D. magna* survival (Fig. 1), but we found no effects for the parasite treatment (no main parasite effect and no parasite x food quality interaction effect; Table 2). In general, the impact of *P. ramosa* or WFCD on host survival was low to absent (Fig. 2). When we zoomed in on the two selected time points (day 14 and day 33), we did find a significant main parasite effect (Fig. 2, Table 2), with individuals infected with *P. ramosa* having a significantly higher survival than individuals from the mixed infection treatment (significant post-hoc difference between means for *P. ramosa* and mixed infection treatment, averaged over both food quality treatments and time points: $p = 0.0018$, $z = -3.59$). This main parasite effect was mainly driven by the significant differences in survival at extreme food limitation at day 14 (Fig. 3B, see further). When survival was considered for these two time points, we found a significant parasite x food quality x time interaction effect (significant $FQ \times P \times T$ interaction in Table 2). For each of these time points, there was no difference in host survival in response to the parasite infection at a low level of food limitation (Fig. 3A, 3C; non-significant post hoc comparisons between each of the parasite treatments exposed to a low level of food limitation). However, at day 33 of the experiment and low food limitation, there was a synergistic effect under multiple parasite exposure, given that the expected survival under the assumption of additive effects was higher than the observed survival (Fig. 3C). When food limitation increased, we did detect a difference in the way parasites impacted host survival. At day 14 and extreme food limitation, individuals infected with *P. ramosa* had a significantly higher survival than did individuals from the mixed infection treatment (significant post-hoc difference between *P. ramosa* and mixed infections for the extreme food limitation treatment at day 14, $p = 0.016$, $z = -3.375$). At this time point and food quality treatment, exposure to both parasites interacted synergistically to decrease host survival, given that the expected survival under the assumption of additive effects was higher than

Figure 1 – Linear predictor given for each *Daphnia magna* individual, in response to the age of *Daphnia* individual (time in days) and the different degrees of food limitation: limited (L, full line) and extremely limited (EL, dashed line). The value of the linear predictor is inversely related to the risk to death, i.e., the higher value for the linear predictor, the lower the risk to death. Error bars represent $\pm 2 \times SE$, $n = 40$. Data for the different time points within the same food quality treatment were considered as repeated measures and were included accordingly in the statistical analysis.

the observed survival under multiple parasite exposure (Fig. 3B). At the final day of the experiment (day 33) and high food limitation, survival was 0% for the WFCD treatment and lower compared to the placebo and the other parasite treatments (Fig. 3D), although post-hoc comparisons between the different parasite treatments for this food quality treatment at day 33 were never significant. Because of the complete mortality in the WFCD treatment, the expected survival under the assumption of additive parasite exposure effects amounted to 0% (Fig. 3D). However, there was no difference in the observed survival in the multiple parasite exposure compared to the placebo and *P. ramosa* treatment after 33 days.

Reproductive output was low during the experiment (total number of juveniles produced per jar in the placebo treatment: 2.8; WFCD: 2.35; *P. ramosa*: 1.52; WFCD + *P. ramosa*: 4.55), but we found a positive effect of parasites on the number of offspring produced and the time to release the first clutch (Table 2, Fig. 4). Hosts exposed to a single parasite species did not reproduce more or at a different time point compared to the placebo treatment; exposure of the host to the two parasites simultaneously did, however, shift the time to commence reproduction to an earlier time point, which increased the number of offspring produced: there was a significant post-hoc effect comparing *D. magna* individuals under multiple parasite exposure versus single *P. ramosa* exposure with respect to the time to commence reproduction and the number of offspring (significant post-hoc comparisons between means for *P. ramosa* and mixed infection treatment, averaged over both food quality treatments, for time to commence reproduction: $z = 2.573$, $p = 0.049$; and for number of offspring produced: $z = -3.041$, $p = 0.012$; Fig. 4A–B). The observed reproductive output in the multiple parasite exposure treatment was significantly higher than expected when considering the single parasite exposure effects (Fig. 4A). Increasing nutrient limitation also impacted the number of offspring produced with *D. magna* individuals reproducing less at high nutrient limitation (from 3.65 ± 0.56 juveniles at low nutrient limitation to 1.92 ± 0.41 juveniles at high nutrient limitation, significant algal culture treatment effect in Table 2). There was, however, no

Figure 2 – Proportion of surviving individuals for the different parasite treatments. Values are mean values over two different time points (day 14 and day 33 of the experiment) and the two food quality treatments (limited and extremely limited food quality). Day 14 was chosen as WFCD is known to exert strong virulence effects from this day and further; day 33 as this was the final day of the experiment. Different letters represent significant post-hoc differences. Past = *Pasteuria ramosa*, WFCD = White Fat Cell Disease, Mix = *Pasteuria ramosa* + WFCD treatment. Error bars represent $\pm 2 \times \text{SE}$, $n = 20$.

Figure 3 – Proportion of surviving individuals at different time points in the experiment (upper panels A and B: survival after 14 days, lower panels C and D: survival after 33 days) and in the different algal treatments (left panels A and C: highest food quality, right panels B and D: lowest food quality – most extreme nutrient limitation). Day 14 was chosen as WFCD is known to exert strong virulence effects from this day and further; day 33 as this was the final day of the experiment. Error bars represent ± 2 SE, $n = 10$. Different letters represent significant post-hoc differences. Open circles represent estimated survival in the combined treatment under the assumption of additive effects of the single parasite treatments (see Material and methods: ‘Prediction of joint effects of parasites’). Past = *Pasteuria ramosa*, WFCD = White Fat Cell Disease, Mix = *Pasteuria ramosa* + WFCD treatment.

Figure 4 (next page) – Reproductive parameters for *Daphnia* related to the parasite treatment. A: Number of offspring produced per jar (each jar containing five individuals). B: Timing to release the first clutch, expressed as the linear predictor. The value of the linear predictor relates to the chance that first reproduction occurs early. Error bars represent ± 2 SE, $n = 20$. Different letters represent significant post-hoc differences. Open circles represent estimated survival in the combined treatment under the assumption of additive effects of the single parasite treatments (see Material and methods: ‘Prediction of joint effects of parasites’). Past = *Pasteuria ramosa*, WFCD = White Fact Cell Disease, Mix = *Pasteuria ramosa* + WFCD treatment.

TABLE 2

Output of statistical analyses on *D. magna* fitness parameters, specific tests are given in Material and methods. “Time to death” refers to the outcome of the survival analysis, “Time point analysis” refers to the outcome of the repeated measures generalized linear model (see Material and methods). Parameters that were not included in the minimal adequate model are indicated by “NA”.

Time to death			
	df	χ^2	p-value
Food quality (FQ)	1	6,99	0,0082
Parasite (P)	3	3,99	0,26
FQ x P	3	0,85	0,84
Time point analysis			
		χ^2	p-value
Food quality (FQ)	1	3,66	0,056
Parasite (P)	3	13,06	0,0045
FQ x P	3	4,67	0,2
FQ x P x Time	4	84,36	< 0.001
Amount of juveniles			
		χ^2	p-value
Food quality (FQ)	1	7,96	0,005
Parasite (P)	1	11,95	0,007
FQ x P	3	NA	NA
Time to first reproduction			
		χ^2	p-value
Food quality (FQ)	1	NA	NA
Parasite (P)	1	9,03	0,03
FQ x P	3	NA	NA

significant interaction between the type of parasite used and the degree of nutrient limitation for both reproductive variables (Table 2).

Discussion

Our study confirms that a reduced food quality negatively affects host reproduction and survival, which is in line with earlier studies focusing on the food quality – host – parasite triad (FROST *et al.* 2008; CORNET *et al.* 2014). Additionally, we here show that food quality negatively affects parasite virulence with respect to host mortality upon multiple compared to single parasite exposure. Although both our food quality treatments were always limiting to a certain degree, we argue that this lack of a good-condition control does not jeopardize our main conclusion, i.e., that hosts suffer strongly under a strong decrease in food quality upon a double infection treatment compared to a single infection treatment. Virulence effects in the highest food quality treatment were small to absent, but parasite-induced effects were more pronounced when nutrient limitation was more severe and/or under multiple parasite species exposure, depending on the considered time point in the experiment. This result is in line with many studies that show that parasite virulence resembles the virulence of the most virulent competitor or increases under co-infection (BEN-AMI *et al.* 2008; SCHMID-HEMPEL 2011; ALIZON *et al.* 2013; GRIFFITHS *et al.* 2015). However, we here show that this especially occurs upon food quality (nutrient) manipulation. However, the higher parasite-induced mortality in the multiple parasite exposure treatment was not reflected in decreased reproduction, given that this treatment was associated with an earlier time to start to reproduce and a higher reproductive output in the *D. magna* host.

Both *P. ramosa* and WFCD are virulent parasites and most likely have a high energy demand to support parasite growth, assuming that the rate at which parasites extract *Daphnia* nutrients equates with their virulence (EBERT *et al.* 2004; SMITH & HOLT 1996; MIDEO 2009; CRESSLER *et al.* 2014). In this study, single parasite species' effects on *Daphnia* survival and reproduction were minor (even positive) for *P. ramosa*. It was mainly WFCD that caused the increased mortality, given that under high nutrient limitation and at day 14 of the experiment, the multiple parasite exposure treatment resulted in a significantly lower survival than did the single *P. ramosa* exposure treatment. Furthermore, WFCD exposure was associated with 100% mortality by the last day of the experiment. By the last day, the observed mortality in the multiple parasite exposure was significantly lower than the expected mortality (100%). Although this appears to contrast at first sight with our expectations of increased virulence in the multiple parasite treatment, this effect is probably due to the mortality effect induced in the WFCD treatment, combined with the low level of variation for the response (given that overall survival was low).

The stronger mortality effect for WFCD than for *P. ramosa* can be linked to a difference in infection and exploitation strategies of the two parasites. Expression of parasite virulence depends on a basic level of energy reserve of the host and the parasite to maintain its growth (SMITH & HOLT 1996; MIDEO 2009; CRESSLER *et al.* 2014). Immunological mechanisms in the host reduce this basic energy level and thus reduce the energy available for the parasite. Simultaneously, parasite loss rates increase with increasing host immunological responses and thus level up the amount of energy needed to induce a successful infection (HALL *et al.* 2009). The low energy level of the *D. magna* individuals might have resulted in a failure to establish an infection for *P. ramosa*. Important to note is that the individuals of *Daphnia* in this experiment were always nutrient limited to some degree, which is reflected in the low overall fecundity. As such, we assume that there was strong resource competition between the hosts and parasites, in which it was difficult for the parasites to exploit host resources and as a result infections did not get well established. However, WFCD is probably a more efficient parasite when energy provision in the environment and in the host is low. WFCD causes strong phenotypic changes in the adipose tissue, while *P. ramosa* infects the hemolymph and sterilizes its host. Affecting the adipose tissue suggests that the parasite boosts the immune system, but at the same time also retrieves energy from it, in both cases a costly process for the host that may be associated with the high parasite-induced mortality.

This may also explain why WFCD always induces field infections early in the season, when food is limited due to fast growing populations of *Daphnia* sp., whereas *P. ramosa* can only establish infections later in the growing season when food is often not limiting (DECAESTECKER *et al.* 2005; SCHOEBEL *et al.* 2014). Equally as for *P. ramosa*, HALL *et al.* (2009) found that the outbreak of a fungal epidemic (*Metschnikowia bicuspidata*) in *Daphnia* was delayed when the food quality in the environment was low. Nevertheless, under non-limited conditions (i.e. where food quantity and quality is high), causing chronic infections and retrieving resources from the reproductive system, as *P. ramosa* does, is also an efficient strategy in natural populations (EBERT *et al.* 2004; DECAESTECKER *et al.* 2005, 2015).

Under the combined infection of *P. ramosa* with WFCD, *D. magna* individuals also shifted their reproduction forward. Some organisms do this when subjected to stressful conditions, in an attempt to increase fitness when resources are not yet completely depleted. Such a reproduction shift has been observed several times in *Daphnia* sp. under stressful conditions, e.g., predation (STIBOR 1992), temperature increase (GEERTS *et al.* 2014), metal contamination (LOPES *et al.* 2004). This “fecundity compensation” has also been identified for *Daphnia* sp.-parasite interactions (CHADWICK & LITTLE 2005; COOPMAN *et al.* 2014; LANGE *et al.* 2014). This can again be linked to an energy allocation perspective. When nutrients are limiting, organisms allocate energy towards increased reproduction and growth rate, but at the cost of survival (BOGGS 2009; PIETRZAK *et al.* 2010).

Conclusions

Understanding the factors that determine and interfere with virulence expression in multiple infections is essential for the prediction and control of infectious diseases. Here, we show that the degree of nutrient limitation drives the outcome of multiple parasite exposure, most probably by interfering with the host stoichiometry, energy allocation and its immune system. As infectious diseases are becoming more prominent, it is important to get more insight into this host-pathogen-food quality interaction, which will help in the understanding and management of disease systems in natural populations. It is essential to consider the complete framework of host and parasite community and food web dynamics (DECAESTECKER *et al.* 2015; AALTO *et al.* 2015). Our study contributes to the understanding of these interactions by indicating that multiple infections induce synergistic effects, but that the effect is dependent upon the parasite species and trait considered.

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