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Rapid change in parasite infection traits over the course of an epidemic in a wild host–parasite population

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By combining a field study with controlled laboratory experimentation, we examined how infection traits of the sterilizing bacterium, *Pasteuria ramosa*, changed over the course of a growing season in a natural population of its crustacean host *Daphnia magna*. The number of parasite transmission spores per infected host increased ten-fold over the course of the season, concomitant with a decline in the density of infected hosts. Plausible explanations for this variation include changes in environmental conditions, changes in host quality, or that parasite migration or natural selection caused a genetic change in the parasite population. We sought to distinguish some of these possibilities in a laboratory experiment. Thus, we preserved field-collected parasite spores throughout the season, and later exposed a set of hosts to a fixed dose of these spores under controlled laboratory conditions. Parasites collected late in the season were more infectious and grew more rapidly than parasites collected early in the season. This result is compatible with the hypothesis that the observed increase in infectivity in the field was due to genetic change, i.e. evolution in the *P. ramosa* population.

Parasites and pathogen populations can change dramatically over very short time periods, as evidenced by the evolution of drug resistance (Marchese et al. 2000), the emergence of vaccine escape mutants (Bangham et al. 1999), the evolution of infectivity (the capacity to infect), the rate of within-host proliferation and the degree to which parasites stimulate the host's immune system (Paterson and Barber 2007). Shifts in infection traits may be due to genetic change in the parasite population, though this will not always be the case. For example, infection-related traits may change when the quality of the host as a resource changes due to shifts in host food quantity (Krist et al. 2004, Vale et al. 2011) and quality (Hall et al. 2009), or when there are changes in environmental factors such as temperature (Blanford et al. 2003, Vale et al. 2011).

We used a model system, the crustacean *Daphnia magna* and its sterilizing bacterial parasite *Pasteuria ramosa,* to both document an intriguing pattern of evolution in the field, and probe for its cause. The *Daphnia–Pasteuria* system has been used extensively to explore how various genetic and non-genetic phenomena affect the incidence and severity of parasitism (Ebert 2008, Auld et al. 2010, 2012a, b, Duneau et al. 2011, Luijckx et al. 2012). Another laboratory study of this system has also provided compelling support for key predictions from the evolution of virulence theory, specifically that parasite virulence is tradedoff with transmission potential (Jensen et al. 2006). These *P. ramosa* infection traits have been examined under controlled laboratory conditions and the relationship between within-host replication and virulence has not been much linked to natural epidemiological and disease severity patterns.

We studied changes in parasite prevalence (the proportion of infected hosts), infection intensities (the number of parasites growing within each infected host) and the number of haemocytes circulating in the host across a growing season in a natural population. These parameters were associated with host population densities. To disentangle whether any observed changes in the field were due to changes in the parasite population or to changes in the complement of host genotypes, we exposed, under common garden conditions in the laboratory, a set of standard (i.e. reference) host genotypes to parasites collected (and then stored frozen) from different times during the field season. Thus we combined observations of parasite change in the field with an experiment that could shed light on possible causes of that change.

Material and methods

Study organisms

Daphnia magna is a cyclically parthenogenetic freshwater planktonic crustacean that inhabits shallow freshwater ponds. *Daphnia* are frequently exposed to and infected with the sterilizing microparasite, *Pasteuria ramosa* (Ebert 2008). *Pasteuria ramosa* is a spore-forming bacterium that is transmitted horizontally from the corpses of previously infected hosts (Ebert et al. 1996). *Daphnia* take in *P. ramosa*

transmission spores along with their food, and once in the host, the *P. ramosa* spores go through a 10–20 day developmental process, resulting in millions of transmission spores that are released upon host death. The process of parasite development and reproduction uses up resources that would otherwise be used for host reproduction, and *Daphnia* are almost always sterilised as a direct result of infection with *P. ramosa*. *Pasteuria*-infected *Daphnia* can be easily identified by eye: they have obvious red bacterial growth in their haemolymph, and are usually larger and lack developed ovaries and eggs in their brood chamber. Whilst *P. ramosa* is known to infect many *Daphnia* and other cladoceran species (Ebert 2005, Duffy et al. 2010, Auld et al. 2012c), in this population *D. magna* is the only available host and is therefore the key agent in shaping *P. ramosa* infection traits.

The *Daphnia–Pasteuria* model has been used in many studies of parasite fitness because infection status is easily determined and transmission stages (henceforth transmission spores) are only released on the death of the host (Ebert et al. 1996). Good estimates of both parasite lifetime reproductive success and transmission potential can therefore be obtained by counting the number of transmission spores from infected hosts (Jensen et al. 2006), and *P. ramosa* spores remain infectious after being frozen at −20°C (King et al. 2013). Also, the number of circulating haemocytes in the host rapidly increases soon after exposure to infectious *P. ramosa* spores (i.e. there is a cellular response: Auld et al. 2010, 2012a, b), and baseline haemocyte number is greater in *P. ramosa*-infected as opposed to healthy hosts (Auld et al. 2012b).

Field haemocyte and parasite spore counts

Daphnia magna were sampled from three fixed points in a pond at Kaimes Farm, Leitholm, Scottish Borders (2°20′43″W, 55°42′15″N) twice per month between April and November 2010. This pond is approximately 500 m from the pond surveyed in a previous field study (Auld et al. 2012b). The pond contains other *Daphnia* species, but *P. ramosa* has only been observed to infect *D. magna.* Adult *D. magna* were collected by sweeping a net with an opening of 0.063 m^2 through one metre of pond water; they were then grouped according to infection status. Hosts from each grouping were placed five at a time in a cell extraction chamber with 4.0 µl of ice-cold anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 17 mM EDTA and 41 mM citric acid, pH adjusted to 4.5: Lavine et al. 2005) and their hearts were pierced using a 25-gauge needle, causing haemolymph to pool into the buffer. This haemolymph–buffer solution was then transferred into 0.5 ml Eppendorf tubes and placed on ice for the hour-long journey back to the laboratory. Cadavers were kept into 1.5 ml Eppendorf tubes.

In the laboratory, each of the haemolymph samples was mixed thoroughly and 2 µl were placed in a fertility counting chamber (0.001 $mm^2 \times 0.100$ mm depth). The number of haemocytes per µl of haemolymph–buffer solution was then determined. The cadavers were homogenized in 500 µl of ddH_2O, and 8 µl were placed in a Neubauer (improved) counting chamber (0.0025 $mm^2 \times 0.1$ mm depth), and the number of *P. ramosa* transmission spores (an estimate of parasite fitness) was determined. These spore solutions were then frozen at −20°C.

At each sampling location, water temperature was measured using a digital field thermometer. The population density of *Daphnia* was estimated by counting the number of infected adults, healthy adults and juveniles. *Pasteuria ramosa* infection was assessed in the adult portion of all subsamples. Infection was usually assessed by eye, but in the occasional ambiguous case, individuals were crushed under a glass coverslip on a microscope slide, and then examined under a transmission microscope for the presence of *P. ramosa* spores.

Experimental setup

We performed two experiments. The first experiment was designed to test if 1) the ability of *P. ramosa* to infect, 2) its reproductive success in infected hosts and 3) its ability to elicit a host cellular response changed over the course of the season. The second experiment was designed to test whether the parasite-mediated mortality (a measure of virulence that strongly influences *P. ramosa* life-history) depended on whether *Daphnia* were infected with *P. ramosa* collected from the beginning or the end of the season.

A test set of four standard *Daphnia* genotypes (named KA40, KA53, KA62 and KA81) were maintained as independent replicates. These host genotypes were chosen because they vary in susceptibility to *P. ramosa* spores from this study population. All four host genotypes were used in the first experiment, and KA53 and KA62 were used in the second experiment. These genotypes were founded from laboratory-hatched ephippia collected from a local pond (500 m from the current study site; Auld et al. 2012b) and kept in the laboratory in a state of clonal reproduction for three generations to minimize variation in condition. Hosts were kept in groups of five in jars containing 200 ml of artificial medium (Klüttgen et al. 1994) and fed 5.0 ABS of chemostat-grown *Chlorella vulgaris* algal cells per day (ABS is the optical absorbance of 650 nm white light by the *C. vulgaris* culture; 5.0 ABS is an abundance of algal food). The jars were incubated at 20°C on a 12L:12D light cycle, and their medium was changed three times per week, and after the *Daphnia* had offspring. Second clutch neonates formed the experimental replicates in each of the two experiments.

The parasite spores used were from samples collected during the field study on 8 June, 6 July, 3 August, 17 August, 2 September and 16 September 2010. These are from the same samples as spores used for the spore counts shown in Fig. 1B, and were frozen (at −20°C) within 3 h of collection. On the day of experimentation, the spore samples were defrosted. Six consolidated spore solutions (one for each date) were then made; they consisted of equal numbers of spores from each replicate sample collected on that specific date. Spore solutions were then diluted with ddH_2O until each final solution was at a concentration of 1×10^6 spores ml^{-1}.

Experiment 1

Each experimental replicate five *Daphnia* consisted in 200 ml of artificial media. Replicates were divided between seven treatments: they were either controls, or were exposed to

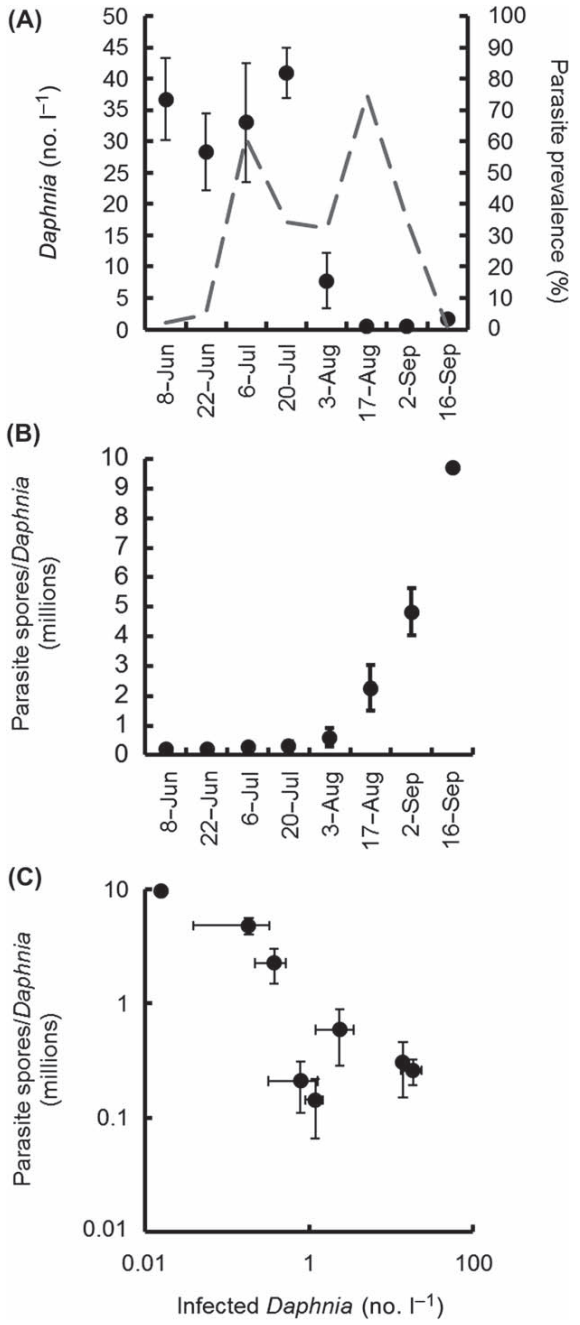

Figure 1. (A) *Daphnia* population density (mean ± 1 SE, bold circles) and mean *P. ramosa* prevalence (grey dashed line) over time; (B) the number of *P. ramosa* transmission spores per infected host (mean ± 1 SE) over time; and (C) the association between the numbers of *P. ramosa* transmission spores per infected host and the density of infected infected hosts (both mean ± 1 SE).

one of the six parasite spore solutions. There were 12 replicates per parasite treatment, per genotype and thus a total of 336 replicates.

Experimental replicates were kept in the same conditions as maternal generations until at least three of the five *Daphnia* deposited eggs in their brood pouch, at which point they were ready for parasite exposure. Parasite treatments were as follows: for each replicate, the five adult *Daphnia* were placed in the well of a 24-well plate. Replicates assigned to the parasite-exposed treatments then received 5×10^4 *P. ramosa* spores (50 µl), and control replicates received an identical volume of homogenized healthy *Daphnia* as a placebo.

Treatment exposure lasted for five hours, after which the *Daphnia* were removed from the cell plate and washed in artificial medium. Four of the five hosts in each replicate were dried on a paper towel and then placed on a glass petri dish. Their hearts were pierced with a 25 gauge needle, and from each of the four *Daphnia*, 1.0 µl of haemolymph was pipetted and mixed with 4 µl of anticoagulant buffer. Haemocytes were then counted using methodology described earlier. It is important to note that this measure of host cellular immune activity in the laboratory is different to the number of haemocytes recorded in the field: the initial haemocyte number in the experiment reflects a hosts response to the initial stages of infection, whereas the number of haemocytes documented in infected samples in the field reflects host cellular immune activity once infection is established. In any case, both measures yield similar information regarding infection in the *Daphnia–Pasteuria* system: an increase in haemocyte number following parasite exposure predicts likely future infection (Auld et al. 2010, 2012a, b).

The fifth *Daphnia* from each replicate was placed singly in a small jar with 60 ml of artificial medium, and medium was refreshed three times per week. The experiment was terminated on day 30, when all surviving hosts were placed in a 1.5 ml Eppendorf and stored at −20°C. Counts of *P. ramosa* transmission spores in each infected host were determined as follows: individual *Daphnia* were homogenized with 100 µl of ddH_2O, and two independent counts were made from the resulting suspension using a Neubauer (improved) counting chamber ($0.0025\ mm^2 \times 0.1$ mm depth).

Experiment 2

We ran a second experiment to better study parasite-induced mortality. Thus, following exposure to the parasites, infected hosts were maintained until they died. Replicates were maintained in the same manner as those used in experiment 1, and exposure protocols were also the same. There were two parasite treatments: hosts were either exposed to *P. ramosa* collected at the beginning of the season (8 July) or at the end of the season (2 September). There were 100 replicates per parasite treatment, per genotype and thus a total of 400 replicates. Each replicate was exposed individually to 5×10^4 spores for 5 h. After the exposure period, replicates were changed into new jars and fresh medium, and medium was then refreshed three times per week. Jars were monitored daily for mortality, and infection status was scored by eye.

Analysis of field data

All data were analysed using general linear models implemented in the R statistical package, and for all models, the significance of the predictor variables was examined hierarchically using a stepwise backward model reduction procedure (Crawley 2007).

First, we examined how ecological variables associated with the numbers of parasite spores from infected hosts. This was done by testing the effects of temperature, $\log_{10}$[infected host density] and $\log_{10}$[number of haemocytes] on the $\log_{10}$-transformed parasite spore counts. Infected host density was used as a proxy for the availability of susceptible hosts. All two-way interactions were also included as explanatory variables and sample location nested within sample date was included as a random effect. We tested the significance of the random effects by removing them from the model and analysing the resulting change in deviance using a likelihood ratio test.

Next, we analysed the number of haemocytes circulating in the host's haemolymph, our measure of host immune activity. This was done by testing the effects of host infection status (infected or not), pond temperature, parasite prevalence and $\log_{10}$[host density] on $\log_{10}$-transformed haemocyte count data; all two-way interactions again also included as explanatory variables and sample site nested within sample date were included as random effects.

Analysis of experimental data

First we analysed parasite infection traits from experiment 1. We analysed the probability of infection by fitting a generalized linear model with a binomial error structure and a logit link function to the infection data (from parasite-exposed hosts only); host genotype, parasite sample and their interaction were fitted as fixed factors. Then, using data from infected hosts only, we analysed the number of parasite transmission spores using a two-way ANOVA with the same model structure.

Next, we examined the host cellular response from experiment 1. This was done using a two-way ANOVA, where host genotype, parasite exposure (exposed or non-exposed control) and their interaction were fitted as fixed factors. Then, using data from parasite-exposed hosts only, we tested whether parasite sample had an effect on haemocyte counts, again using a two-way ANOVA, but with host genotype, parasite sample and their interaction fitted as fixed factors. In all cases where the data were non-orthogonal, type 3 sums of squares were used.

Second, we examined the host survival data from experiment 2. Specifically, we tested whether host survival depended on whether they were exposed to *P. ramosa* spores from the beginning or the end of the season. This was done using a Cox's proportional hazards analysis applied to data from infected hosts only, where host genotype and parasite sample fitted as fixed factors. All data used in these analyses are archived at Dryad (DOI: 10.5061/dryad.p5k95 Data files: AuldetalOikos).

Results

Field data

Pasteuria-infected *Daphnia* were observed from early June until mid-September, during which pond temperatures varied between 12.1°C and 18.5°C. Parasite prevalence (the proportion of hosts that became infected) peaked twice: in early July and in mid August (Fig. 1A). The number of parasite transmission spores per infected host increased dramatically over the season ($\chi^2 = 6.56$, $p < 0.001$): infected hosts collected in late September had over ten-fold more spores than those collected in early June (Fig. 1B). Sample site within the pond did not explain a significant proportion of variation in the data ($\chi^2 = 7.11 \times 10^{-15}$, $p = 0.99$). After testing the effects of infected host density, infected host haemocyte number and pond temperature (and all two-way interactions) on the number of *P. ramosa* transmission spores, only infected host density remained significant after model reduction: *P. ramosa* transmission spore count was negatively associated with the density of infected hosts ($\log_{10}[y] = 5.76–0.49\log_{10}[x]$, $t_{12} = 3.49$, $p < 0.01$; Fig. 1C).

Finally, confirming previous work (Auld et al. 2012b), parasitized *Daphnia* had consistently more circulating haemocytes than their healthy counterparts ($F_{1,7} = 155.26$, $p < 0.0001$), but haemocyte number was not associated with pond temperature, parasite prevalence or host density. Further, haemocyte number did not vary over the course of the season ($\chi^2 = 0.19$, $p = 0.66$) or across sample sites ($\chi^2 = 6.40 \times 10^{-10}$, $p = 0.99$).

Experiment 1

Pasteuria ramosa collected at the end of the season was more infectious than *P. ramosa* collected earlier (Table 1, Fig. 2), and the final number of *P. ramosa* transmission spores per infected host also depended on the host genotype and the parasite sample date (Table 2, Fig. 3). Further, *Daphnia* mounted a cellular response to *P. ramosa*: parasite-exposed hosts had 1282 ± 53 haemocytes, whereas unexposed controls had 604 ± 43 haemocytes (Table 1). The strength of this cellular response also depended on the specific combination of host genotype and parasite sample date (Table 1), and the number of circulating haemocytes mirrored the proportion of infected hosts in a particular treatment (Fig. 2).

Table 1. The effects of host genotype and parasite sample date on the probability of infection and host cellular immune response.

	DF	LR-χ^2	p
Probability of infection (parasite-exposed hosts)			
Host genotype	3	0.70	0.87
Parasite sample	6	133.83	<0.0001
Post genotype × Parasite sample	18	16.75	0.54
Error	278		
	DF	**F**	**p**
Log-$_{10}$[haemocytes] (all hosts)			
Host genotype	3	3.57	<0.05
Parasite exposure	1	27.02	<0.0001
Host genotype × Parasite exposure	3	2.11	0.10
Error	328		
Log-$_{10}$[haemocytes] (parasite-exposed hosts)			
Host genotype	3	6.16	<0.001
Parasite sample	5	22.80	<0.0001
Host genotype × Parasite sample	15	2.03	<0.05
Error	263		

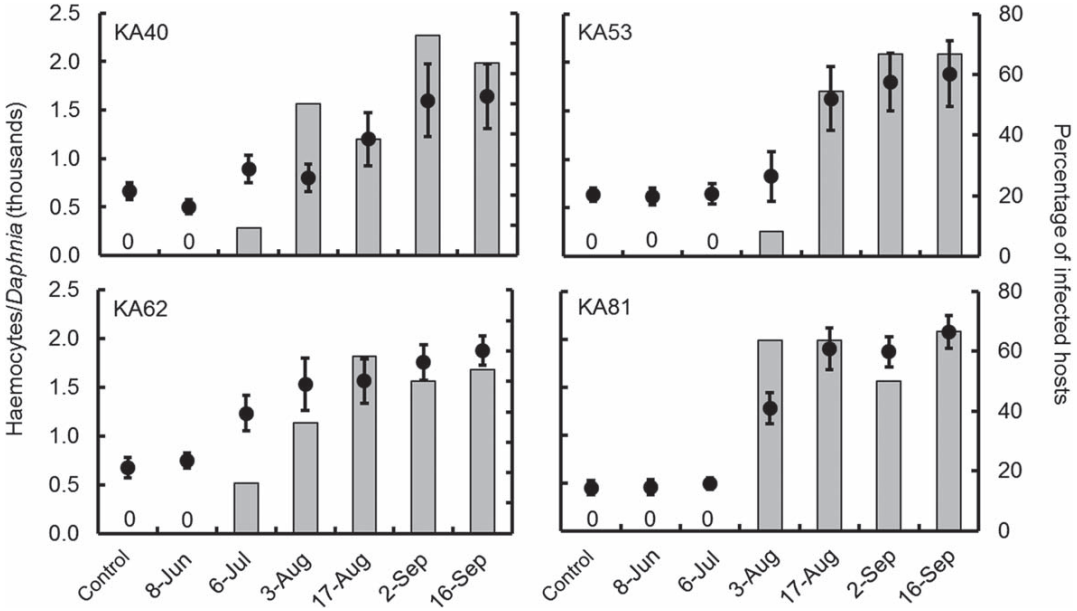

Figure 2. The proportion of infected *Daphnia* (bars, right axis) and the number of haemocytes per microlitre (mean ± 1 SE, circles, left axis) in four host genotypes exposed to parasite samples from a wild population. Zeroes indicate treatments where no infections occurred.

Experiment 2

Again, *P. ramosa* from the end of the season caused more infections than *P. ramosa* from the beginning of the season ($\chi^2 = 64.36$, $p < 0.0001$). Survival of infected hosts, the focus of this experiment, differed between the host genotypes: KA53 hosts survived 48.00 ± 1.25 days whereas KA62 hosts survived 54.38 ± 1.42 days ($\chi^2 = 9.81$, $p < 0.01$). The data also suggest *P. ramosa* collected at the end of the season kill their hosts later than *P. ramosa* collected at the beginning of the season (though this trend is not significant at the 0.05 level): hosts infected with spores collected on 8 July survived 50.20 ± 1.08 days, whereas hosts infected with spores collected on 2 September survived 52.92 ± 2.29 days ($\chi^2 = 3.68$, $p = 0.06$).

Discussion

Our survey of a wild population revealed substantial fluctuation in the prevalence of the bacterium *P. ramosa*, including two peaks where 60–70% of their *Daphnia* hosts were infected. The number of *P. ramosa* transmission spores within infected hosts was 10 times greater in the autumn than in late spring and, concomitant with this increase in spore numbers, the density of infected hosts declined dramatically. Haemocyte counts were also higher in parasitized *Daphnia* than in their healthy counterparts, consistent with an earlier study (Auld et al. 2012b).

Table 2. The effects of host genotype and parasite sample date on the number of parasite transmission spores in infected hosts.

	DF	F	p
Log_{10}[transmission spores] (infected hosts)			
Host genotype	3	14.28	< 0.0001
Parasite sample	4	7.20	< 0.0001
Host genotype × Parasite sample	10	1.01	0.44
Error	85		

These shifts in infection traits may be due to genetic changes in the parasite population, but they may also be due to unmeasured environmental changes in the pond or to demographic changes in the host population. For example, infected hosts collected late in the season could simply be older hosts that have been infected for longer and thus have allowed more time for *P. ramosa* proliferation. In this case, we might not expect parasites collected late-season to differ from those collected earlier. To shed light on this, we brought field-collected parasites into the laboratory throughout the season and preserved them frozen until we could perform an experiment. This experiment addressed whether parasites from different time points expressed different trait values in a 'common garden', where the parasite samples were exposed to a standard set of host genotypes and infection duration and dose was the same for all parasitized hosts. The experiment showed that parasite infectivity, capacity for immunostimulation and within-host growth depended on the date when the parasite spores were initially collected from the wild (Table 1, 2).

These combined field and laboratory observations of phenotypic change in the parasite population are compatible with the hypothesis of genetic change (i.e. evolution) in the parasite population. This could be in response to host evolution (often observed in response to parasitism in *D. magna* populations, Duncan and Little 2007), or because genetically different parasite migrants (either from another population or from the parasite 'seed bank' in the pond sediment) entered the population. The genetic diversity of parasites within each infected host could also play a role in the observed patterns.

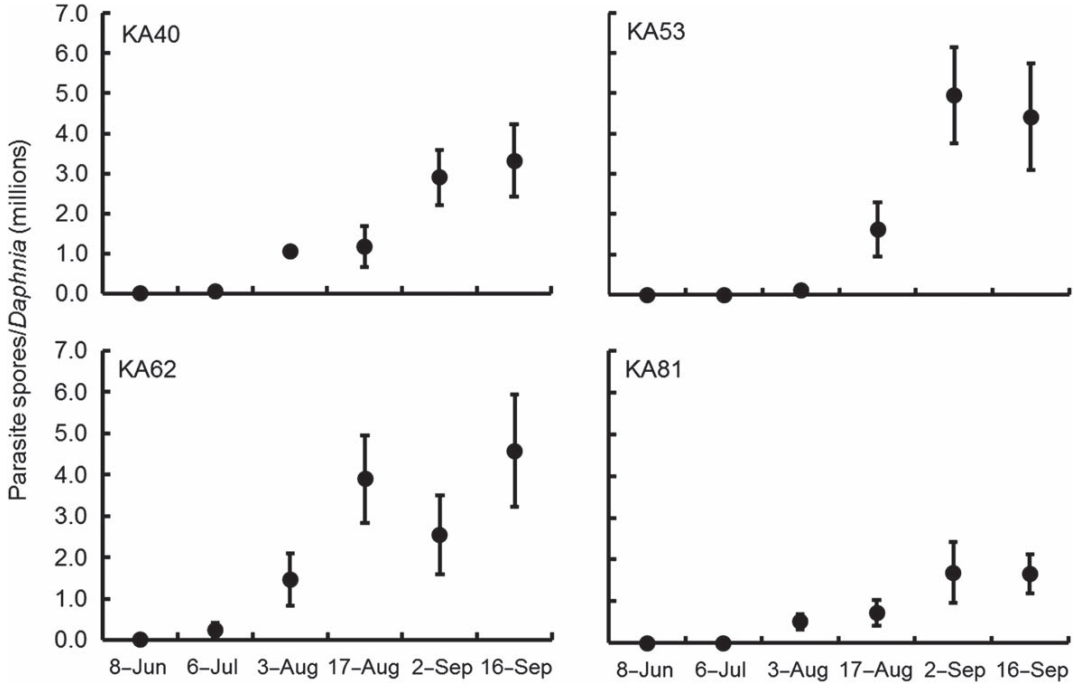

Figure 3. Number of *P. ramosa* transmission spores per infected host (mean ± 1 SE) in four host genotypes exposed to parasite samples from a wild population.

For example if parasite within-host diversity were lower at the end of the season, this could alter within-host competition, with knock-on effects on virulence.

However, a set of explanations that do not rely upon genetic change in the parasite population cannot be excluded. For example, if hosts from the end of the growing season provided a higher quality environment for the parasites, the resulting transmission spores (which we collected and stored) could have been of higher quality, including superior transmissibility, yet genetically identical to early-season spores. A previous study of the *Daphnia–Pasteuria* system found that the amount of food consumed by hosts appears to affect parasite transmission spores and infection related traits on future hosts: well-fed hosts produced more virulent spores (i.e. spores that killed their hosts earlier: Little et al. 2008). Our findings are consistent with this as we found that parasites collected at the end of the season (when food quantity is presumably much lower) tended to be less virulent. However, that earlier study also found the quantity of food consumed by the host had no effect on the infectivity of parasite spores on future hosts, whereas we found a marked increase in parasite infectivity over time.

If natural selection has played a role in the rapid changes in parasite traits, it is intriguing that these traits were negatively correlated with infected host abundance (Fig. 1C). Theory on the relationship between density and virulence predicts that high availability of hosts should favour high parasite growth, virulence and transmission (Bull and Levin 1994, Ewald 1994, Day and Gandon 2007), and our observations clearly do not fit this. This lack of fit is perhaps not surprising since *P. ramosa* infects from its diapausing stage, and standard theory considers directly horizontally transmitted parasites. Parasites with long-lived externally viable stages are predicted to cause high virulence irrespective of transmission opportunities because the relative cost of virulence will be low, assuming the costs of waiting to infect are also low (i.e. when there is little degradation in spore infectivity over time: (Ewald 1994, Bonhoeffer et al. 1996, Walther and Ewald 2004). Given the robust ability of *P. ramosa* to 'wait' for hosts (Decaestecker et al. 2007), we would expect them to evolve consistently high virulence. Thus, theory on 'sit and wait' strategies also does not explain the observed change in parasite growth rate across a season. Clearly, *P. ramosa*'s capacity for diapause, both in terms of natural selection on virulence, and for 'migration' from the seed bank, and will need to be considered further.

Improving our understanding how parasites change over the course of epidemics in wild host populations is important for both pure and applied questions in evolutionary biology. Our findings point towards the evolution of increased parasite infectivity. However, parasite within-host growth, virulence and capacity for immunostimulation, amongst other factors we have not speculated upon here, could have changed as a result of both genetic and non-genetic change in the parasite population. Our findings thus highlight the need to go back into the wild and increase the understanding the natural history of host–parasite systems.

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