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# Heterozygosity and parasite intensity: lung parasites in the water frog hybridization complex

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## SUMMARY

In hybridogenetic systems, hybrid individuals are fully heterozygous because one of the parental genomes is discarded from the germinal line before meiosis. Such systems offer the opportunity to investigate the influence of heterozygosity on susceptibility to parasites. We studied the intensity of lung parasites (the roundworm *Rhabdias bufonis* and the fluke *Haplometra cylindracea*) in 3 populations of water frogs of the *Rana lessonae-esculenta* complex in eastern France. In these mixed populations, hybrid frogs (*R. esculenta*) outnumbered parental ones (*R. lessonae*). Despite variation in parasite intensity and demographic variability among populations, the relationship between host age and intensity of parasitism suggests a higher susceptibility in parentals than in hybrids. Mortality is probably enhanced by lung parasites in parental frogs. On the other hand, while parental frogs harboured higher numbers of *H. cylindracea* than hybrid frogs, the latter had higher numbers of *R. bufonis*. Despite such discrepancies, these results support the hybrid resistance hypothesis, although other factors, such as differences in body size, age-related immunity, differential exposure risks and hemiclinal selection, could also contribute to the observed patterns of infection.

Key words: hybrid, hybridogenesis, water frogs, *Rana* species, lung parasites, *Rhabdias bufonis*, *Haplometra cylindracea*.

## INTRODUCTION

Because hybridization produces innovative genetic combinations by joining two different genomes, performance analysis of hybrid individuals may contribute to the study of host-parasite coevolution (Mouliat *et al.* 1995; Arnold, 1997). The current concepts about the evolution of hybrid-parasite systems do not, however, allow the proposal of monolithic hypotheses, probably because of the great diversity of genetic associations covered by the term 'hybridization' (from subspecies to genus). While hybridization between phylogenetically distant genomes is expected to promote high susceptibility to the parasite, because of breakdown of genetic coadaptation, hybridization between closely related taxa could enhance resistance through a heterosis effect (Sage *et al.* 1986; Dupont and Crivelli, 1988; Whitman, 1989; Mouliat, 1999). The hypotheses established by Fritz *et al.* (1999) for predicting the impact of herbivory in plants provide an initial theoretical framework for the study of parasite-hybrid systems. Our aim was to focus on the

divergence between susceptibility and resistance, taking advantage of the hybridogenetic system of the water frogs of the *Rana lessonae-esculenta* complex. In a hybridogenetic complex, the genome of one parental species carries distorter genes, which induce the elimination of the genome material of the other parental species before meiosis in the germ line of hybrids (Schultz, 1969; Tunner 1974; Joly 2001). The distorter genome is then duplicated and the gametes of hybrid individuals carry only the genome of one of the parental species. These hybrids usually breed with the taxon whose genome is eliminated, thus restoring the hybridness of their descent (sexual parasitism). In the hybrid lineages, the distorter genome is transmitted clonally (hemiclones). Because of the lack of recombination between the parental genomes (*cf* Pagano and Schmeller, 1999), hybrids are genetically similar to F1 hybrids. In one of the water frog complexes (*R. lessonae* [LL genome] and its hybridogen *R. esculenta* [RL genome], which carries a *ridibunda* [R hemigenome]), the success of hybrid lineages has been widely analysed; several studies (Tunner and Nopp, 1979; Berger and Uzzell, 1980; Semlitsch, 1993, Hotz *et al.* 1999) support the hypothesis of heterotic advantage with respect to fecundity, predation avoidance or hypoxic tolerance of froglets (heterosis effect). However, other studies (Pagano *et al.* 2001; Plénet *et al.* 2000a,b,

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2005; Negovetic *et al.* 2001) support a simple additive hypothesis with regard to habitat use, hypoxic tolerance of tadpoles and thermal tolerance (intermediate performances of the hybrids). The water frog hybridization complex thus makes it possible to investigate *in situ* the relationship between hybridization and parasite infection by comparing the infection rates of 2 taxa (the hybrid *Rana esculenta* and the sexual host *R. lessonae*) that occur concomitantly in the same habitats (*Rana ridibunda* usually does not occur in the same habitats as *R. lessonae*). Water frogs host a great diversity of parasites (Walton, 1949; Saglam and Arikan, 2006) of which we studied the infection with lung parasites *Rhabdias bufonis* (Nematoda) and *Haplometra cylindracea* (Trematoda). In the present study, our aim was to compare the intensity of parasite infection between the two taxa of 3 populations occupying fishponds in eastern France. We took advantage of the possibility of determining individual ages by means of skeletochronology, in order to analyse the relationships between age, mortality and parasite accumulation.

## MATERIALS AND METHODS

### *Study sites*

The frogs were sampled on the Dombes Plateau (north-east of Lyon, France), where 1000 fishponds devoted to growing carp are distributed over a 900 km<sup>2</sup> area. Water frogs of the LE hybridogenetic complex occur in high densities at the banks of these ponds. We sampled the frogs at 3 ponds: Boufflers pond (designated as POND A) which is within the estate of the Pierre Vérots Foundation, Saint-Jean-de-Thurigneux (4°51'E-45°56'N), La Tille pond (POND B) at Saint-André-le-Bouchoux (5°55'E-46°59'N) and a third pond (POND C) of the Regional Research Institute for Aquaculture (IRRA) at Saint-Nizier-le-Désert (5°10'E-46°04'N). Because the dispersal range of the water frogs is usually less than 2 km (Holenweg-Peter, 2001), the present populations can be considered independent, as they are separated from one another by more than 15 km (i.e. 15 km between POND B and POND C and 30 km between POND A and both POND B and POND C).

### *Sampling, taxon identification, age estimation and measurements*

We decided to focus on females because of their crucial importance in population dynamics. We sampled frogs from the end of April to the end of May during the breeding period. They were caught by hand during the night using spotlights. We sampled the whole perimeter of each pond. Juvenile frogs (<40 mm) and males were directly released,

whereas females were frozen until performing tissue sampling and measurements. Length from snout to urostyle was measured to the closest 0.1 mm using callipers. We measured body mass after removing the ovaries, which were weighed separately. We estimated body condition by the residuals of the regression of body mass on body length, both measurements being converted into natural logarithms. This index was used because it was shown to be independent of the population under consideration (Jakob *et al.* 1996). Body condition was defined independently for each genotype, because the relationship between body mass and length was considered to vary according to the genotype considered. We identified parental species from hybrids by specific allozymic markers detected by protein electrophoresis of finger tissue using lactate dehydrogenase (LDH-B; EC 1.1.1.27) and mannose-6-phosphate isomerase (MPI; EC 5.3.1.8) (Beerli, 1994; Pagano *et al.* 1997). We estimated individual age by skeletochronology performed on finger bones (Castanet *et al.* 1977; Augert and Joly, 1993).

### *Lung parasites*

We elected to specifically study lung parasites after performing a preliminary analysis of the parasite intensity of the whole body (40 frogs examined). The parasite fauna was mainly composed of roundworms and flukes in the digestive tract and in the lungs. Because lung parasites (*Rhabdias bufonis* and *Haplometra cylindracea*) were frequent and their damage to lung tissues was expected to be more severe than that of gastrointestinal parasites, we focussed on them. While we identified the roundworm as *Rhabdias bufonis*, the recent discovery of new species identified in North and Central America (Tkach *et al.* 2006; Kuzmin *et al.* 2007) suggests that the taxonomy of the genus *Rhabdias* needs to be revised. However, we decided to use this taxon name until further systematic and phylogenetic investigations have been performed in Europe. We extracted the parasites from the lungs by dissection under a stereoscopic microscope. They were then fixed on microscope glass slides using lactophenol for further examinations. We calculated parasite occurrence (presence of parasite), parasite intensity (total number of parasites; Bush *et al.* 1997), and parasite diversity (0, 1 or 2 species).

### *Data analysis*

In order to describe population structure and detect variability between populations, we first compared the structures of the three populations (hybrid proportions, age, body condition and parasite intensity). Hybrid proportions were compared using  $\chi^2$ . Age structures were analysed by using a proportional-odds model, which is a generalized linear

model that relates an ordinal response variable (here age) to predictor variables (MacCullagh and Nelder, 1989; Guisan and Harrell, 2000). The procedure of model selection is described in the following. We first adjusted all possible models, including 1 or 2 of the tested predictor variables, and their interactions. Tests of the effects of variables or interactions were done by use of the likelihood ratio test (LRT) based on the difference in deviance between 2 nested models, which follows a chi-square distribution (Agresti 1990). All variables or interactions that tended to influence the variable considered ( $P < 0.2$  using the LRT) were combined into a single model, from which redundant terms were finally removed. The goodness-of-fit of the selected model was assessed through analysis of deviance residuals as well as comparison of the residual deviance with a chi-square distribution. Finally, we interpreted the selected model using the parameter estimates and their confidence intervals (Agresti, 1990). To compare body condition among populations, we used linear models and ANOVA after assessing the normality of body condition by visual inspection of observed *versus* theoretical quantiles (Venables and Ripley, 2002). We analysed parasite intensity because it was expected to be more informative than simple presence-absence. For this purpose, we used a negative binomial model, which is considered appropriate for parasite distributions (Wilson *et al.* 1996). Here, the negative binomial distribution was also probable, due to the high variance-to-mean ratio. Negative binomial models relate the number of parasites found in a given individual to the predictor variables, considering that the number of parasites follows a negative binomial distribution (Lawless, 1987). We first tested whether the observed distribution fitted a negative binomial distribution by adjusting the null (i.e. constant) negative binomial model to obtain a maximum-likelihood estimate of the dispersion parameter  $\hat{\theta}_1$  and then by comparing the observed distribution with the expected distribution using visual inspection of the observed versus theoretical quantiles. We also estimated the dispersion parameter using the classical estimate from the mean  $\bar{x}$  and variance  $s^2$  of the data set:

$$\hat{\theta}_2 = \frac{\bar{x}^2}{s^2 - \bar{x}}$$

to ensure that no discrepancy occurred between  $\hat{\theta}_1$  and  $\hat{\theta}_2$  (Wilson *et al.* 1996). Then we adjusted all negative binomial models combining the tested predictor variables and their interactions, always assuming that the dispersion parameter equalled  $\hat{\theta}_1$  to obtain comparable models. Model selection and analysis were performed using the procedure described above for proportional-odds models.

Because population structures differed (see Results section), the dynamics of parasite infection

was expected to vary among sites, thus parasite intensity was analysed separately in each population. We tested the effects of 4 predictor variables and their interactions since the aim of the study was to investigate the influence of genotype (hybrid or parental) on parasite intensity. For each parasite species, we also considered the effects of age, body condition and intensity of the other parasite. Age was studied in order to search for density-dependent patterns, a peaked age-parasite intensity relationship being supposed to reveal parasite-induced host mortality, parasite mortality or age-dependent immunity (Anderson and Gordon, 1982; Hudson *et al.* 2002). With this aim, age was considered both as a linear trend and as a second-order polynomial, the second-order term being searched for in order to detect a peak in the age-versus-intensity relationship. Finally, the relationship between parasite intensity and host body condition was tested to assess parasite infection capacity (Jakob *et al.* 1996). If a parasite is pathogenic, individuals harbouring numerous parasites are expected to exhibit poor body condition, unless the parasite is so pathogenic that infected hosts die and are thus not observed. We first searched for possible relationships between the variables used as predictors of parasite intensity. This preliminary analysis was designed to search for co-linearity between predictor variables. We did not test the relationship between body condition and genotype because body condition was defined independently for each genotype. We tested the relationship between genotype and age using a proportional-odds model. The relationship between age and body condition was tested using a linear regression model and ANOVA. These tests were performed separately in each population. To analyse which factors might be associated with parasite intensity, we first estimated the dispersion parameter for each population and verified that the observed distribution was in accordance with a negative binomial distribution. Then model selection and analysis were performed as described for proportional-odds models. All statistical analyses were done with the R software (Venables and Ripley, 2002), considering an error risk of 0.05.

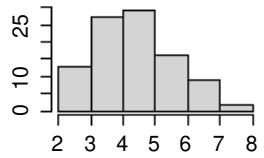
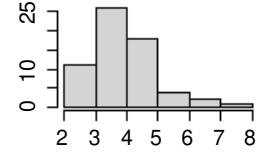
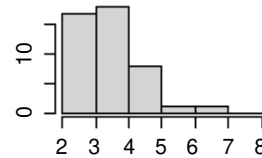
## RESULTS

### *Population parameters*

Table 1 summarizes population structures and describes parasite distribution in each of the three populations. Since age was not available for all the individuals, the sample size was reduced when studying age. The proportion of hybrids appeared to be higher at POND C than at the other sites. Whereas the difference was not significant when considering the three sites separately ( $\chi^2 = 4.22$ , D.F. = 2,  $P = 0.1211$ ), it became significant when

Table 1. Population structure and description of parasite distribution in the three populations

(The relationships between age and genotype and between age and body condition were tested by generalized linear models. For each parasite, 2 estimates of the dispersion parameter are given:  $\hat{\theta}_1$  is the first estimate (see text for definition) and  $\hat{\theta}_2$  is the maximum-likelihood estimate obtained by fitting a null negative binomial model. Only 2 individuals from POND C were infected by *R. bufonis*, thus this parasite was not further studied in this population.)

	POND A	POND B	POND C
Sample size	112	91	53
With age available	96	62	45
Age structure			
<i>Y = Numbers</i> <i>X = Years</i>			
Hybrids (%)	74.1	72.5	86.8
Age-genotype relationship	$P=0.4919$	$P=0.3666$	$P=0.1299$
Age-body condition relationship	$P=0.0525$	$P=0.2507$	$P=0.0227$
<i>H. cylindracea</i>			
prevalence (%)	39.29	65.93	47.17
mean intensity	4.25	3.78	4.48
$\hat{\theta}_1$	0.26	0.60	0.34
$\hat{\theta}_2$	0.24	0.63	0.30
<i>R. bufonis</i>			
prevalence (%)	45.54	25.27	3.77
mean intensity	2.94	3.35	Not estimated
$\hat{\theta}_1$	0.39	0.16	Not estimated
$\hat{\theta}_2$	0.41	0.16	Not estimated
Prevalence of double infection (%)	21.43	16.48	Not estimated

pooling POND B with POND A ( $\chi^2=4.15$ , D.F. = 1,  $P=0.0415$ ). Age structure did not significantly differ between taxa, but it differed among populations, all three differences being significant (POND A against POND C, t-value=2.47,  $P=0.0139$ ; POND A against POND B: t-value=2.43,  $P=0.0151$ ; POND B against POND C: t-value=4.62,  $P<0.0001$ ). Frogs were, on average, oldest in POND A while they were youngest in POND C (Table 1). Conversely, mean body condition did not differ among populations (F-value=0.6341,  $P=0.5313$ ). Whereas the prevalence of *H. cylindracea* was highest in POND B, its intensity did not vary among populations (LRT=2.55, D.F. = 2,  $P=0.2880$ ). In contrast, the number of *R. bufonis* differed among populations (LRT=27.28, D.F. = 2,  $P<0.0001$ ) mainly because this species was rare at POND C. The prevalence of *R. bufonis* was highest in POND A. Taken together, these results show that the three populations had distinct demographic parameters with the lowest apparent mortality at POND A and the highest at POND C where it was related to both a high proportion of hybrids and to the fact that *R. bufonis* was almost absent.

Age structures were not related to genotype (*LL* versus *RL*) in any of the three populations

(Table 1). At POND C particularly, survival of *RL* individuals appeared higher than that of *LL* ones, but the difference was not significant. In this population, age explained a significant part of body condition since body condition decreased with age (Table 1). This relationship supports the hypothesis of high adult mortality in that population. The tendency of decreasing body condition with age was also close to the chosen significance level at Pond A, while it was clearly not significant at POND B (Table 1).

#### Intensity of *H. cylindracea* infection

While the selected model varied from one population to another, the genotype was always involved as a significant variable (Table 2). The residual deviance of the selected models followed a chi-squared distribution for POND B and POND C, but showed data underdispersion for POND A, thus suggesting that individuals were more similar than expected. The deviance residuals failed, however, in detecting any particular structure. The POND A population was the only one in which the intensity of *H. cylindracea* was related to body condition (LRT=2.521, D.F. = 1,  $P=0.044$ ). *H. cylindracea* intensity was

Table 2. Model selection for the intensity of *Haplometra cylindracea* and *Rana bufonis* infection in the three populations

	Variables related to parasite load (P-value of LRT)	Model selected, residual deviance (residual D.F.)
<i>H. cylindracea</i> in POND B	Age (0.159) <i>R. bufonis</i> (0.125) Genotype * <i>R. bufonis</i> (0.032) Body condition * age <sup>2</sup> (0.177)	Genotype * <i>R. bufonis</i> 87.66 (87)
<i>H. cylindracea</i> in POND C	Body condition (0.032) Age (0.086) Genotype * age (0.044)	Genotype * age 31.33 (41)
<i>H. cylindracea</i> in POND A	Body condition (0.007) Genotype (0.013) Age (0.154) Genotype * age (0.007) Body condition * age (0.141) Body condition * <i>R. bufonis</i> (0.192) <i>R. bufonis</i> * age <sup>2</sup> (0.134)	Genotype * age + body condition 62.90 (91)
<i>R. bufonis</i> in POND B	Genotype (0.096) <i>H. cylindracea</i> (0.171) Genotype * <i>H. cylindracea</i> (0.010) Genotype * age <sup>2</sup> (0.063) Body condition * age <sup>2</sup> (0.109) <i>H. cylindracea</i> * age (0.176)	Genotype * <i>H. cylindracea</i> 44.83 (87)
<i>R. bufonis</i> in POND A	Genotype (0.187) Body condition * age (0.052)	Null 98.65 (111)

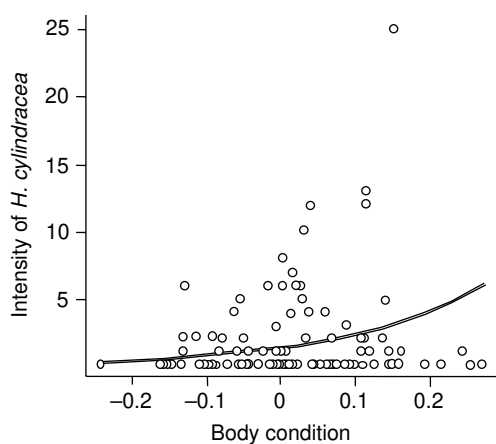


Fig. 1. Relationship between body condition and the intensity of *Haplometra cylindracea* in pond A. Observed data (circles) and predictions of the model including the effect of body condition alone (line).

indeed the lowest in individuals experiencing the lowest body condition (Fig. 1).

At both POND C and POND A, the interaction between genotype and age was significant (POND C: LRT=3.92, D.F.=1,  $P=0.044$ ; POND A: LRT=4.767, D.F.=1,  $P=0.006$ ). In these two populations, the intensity of *H. cylindracea* increased with age in *RL* frogs (Fig. 2). In contrast, the pattern exhibited by *LL* frogs differed between populations. At POND A, the intensity of *H. cylindracea* decreased

as the individuals got older. Conversely, the intensity of *H. cylindracea* increased with age in parental frogs at POND C, but no individual older than 3 years was observed there. Fig. 2 shows that the intensity of *H. cylindracea* in 3-year-old *LL* individuals was very high. Taking into account the results for POND A, we hypothesize that *LL* individuals with high parasite intensity die, so that no individual older than 3 years was observed. Finally, in these two populations, the interaction between age and genotype suggests that parental individuals were more susceptible to *H. cylindracea* than hybrids. At POND B, the selected model was (genotype \* intensity of *R. bufonis*). The only significant factor was the interaction (LRT=4.849, D.F.=1,  $P=0.032$ ). The number of *H. cylindracea* tended to increase with the number of *R. bufonis* in parental individuals, whereas hybrids with many *R. bufonis* harboured few *H. cylindracea* (Fig. 3).

#### Intensity of *R. bufonis* infection

Since only 2 individuals were infested at POND C, we analysed the intensity of *R. bufonis* only in the populations of POND A and POND B. The selected model for POND B showed underdispersion, while the best fit was obtained with the null model for POND A (Table 2). At POND B, the selected model was (genotype \* *H. cylindracea* intensity), thus converging with the results obtained for *H. cylindracea*.

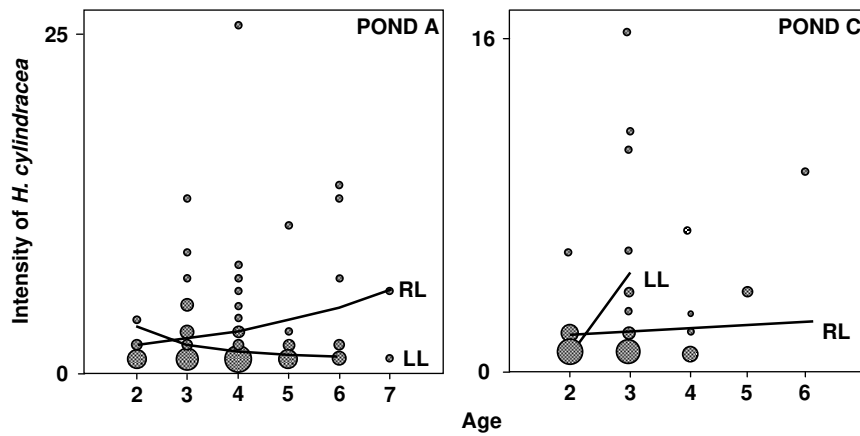


Fig. 2. Relationship between age and intensity of *Haplometra cylindracea* in POND A and POND C populations. Observed data (circles) and predictions of the selected models (lines). LL: parental *Rana lessonae* frogs; RL: hybrid *R. esculenta* frogs. The size of the circles is proportional to the number of individuals observed. Largest circle:  $n=18$  at POND A,  $n=11$  at POND C.

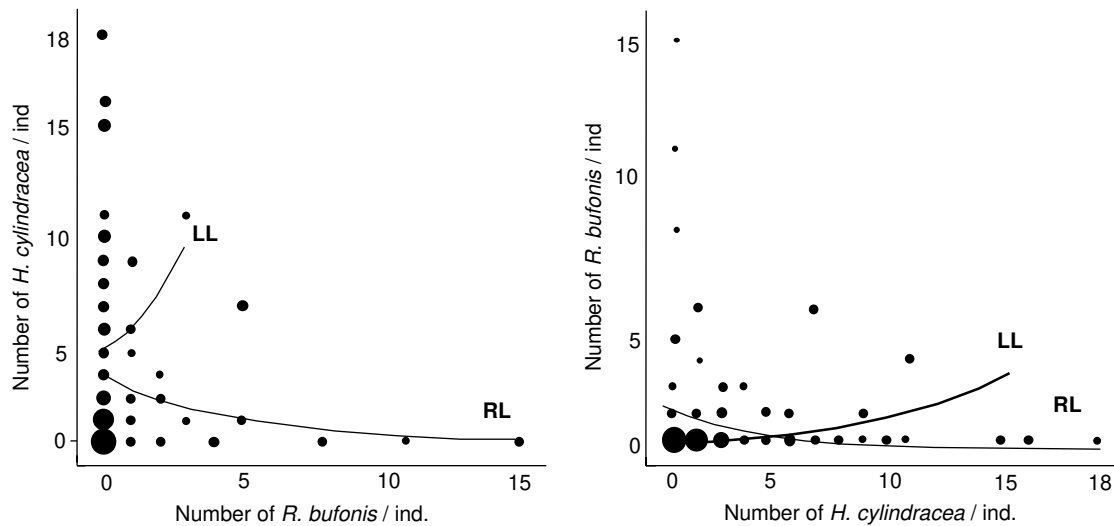


Fig. 3. Relationship between intensity of *Haplometra cylindracea* and *Rana bufonis* in the water frogs of the POND B population, and interaction with genotype. Left: *H. cylindracea* as a function of *R. bufonis*. Right: *R. bufonis* as a function of *H. cylindracea*. Dots are observed data and lines are predictions of the selected models. Dot size is proportional to frog numbers with largest dots = 23 frogs. LL = *Rana lessonae* parental species. RL = *R. esculenta* hybrid.

In this population, parental individuals carrying numerous *H. cylindracea* also harboured many *R. bufonis*. Conversely, hybrids with many *H. cylindracea* had few *R. bufonis*, and individuals with many *R. bufonis* had few *H. cylindracea* (Fig. 3).

#### Variation between populations

Because the pattern in POND B population differed from those in the other populations, we performed complementary analyses to understand which environment or population factors could explain this difference. First, we tested whether parasite incidence differed among populations by comparing the number of *H. cylindracea* in 2-year-old frogs among populations, using a proportional-odds model. The

difference was significant (LRT = 8.35, D.F. = 2,  $P=0.0154$ ). Whereas young frogs carried an average of 4.36 parasites at POND B, the numbers were 0.59 at POND C and 0.38 at POND A. This result lead us to assume that parasite-induced mortality may occur in hybrids as well as in parental frogs in this population, but that the age at peak prevalence may be < 2 years. To obtain further indications, we compared the changes in parasite intensity and variance-to-mean ratio (VMR) with age in the three populations (Fig. 4, VMR was calculated only in samples where  $n \geq 5$ ). The pattern in parental frogs was inconsistent, probably due to low sample sizes. In RL frogs, the number of *H. cylindracea* increased with age at POND A and POND C but decreased at POND B, suggesting that parasite-induced

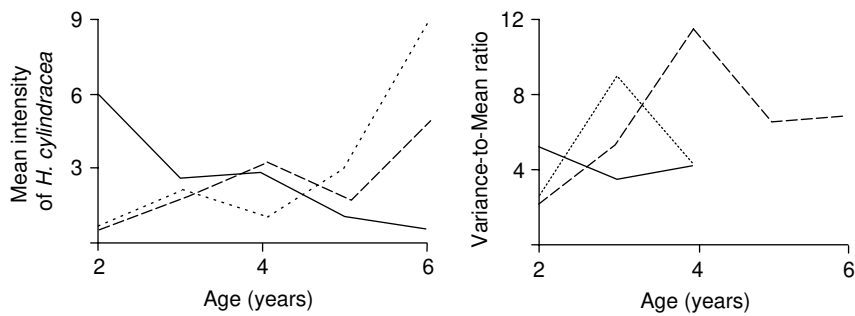


Fig. 4. Mean parasite intensity (left) and variance-to-mean ratio (right) of *H. cylindracea* in hybrid frogs of the three populations. Plain line: POND B population; dashed line: POND A population; dotted line: POND C population.

mortality was much higher in this population. Moreover, VMR showed a peak at POND A in 4-year-old frogs, and at POND C in 3-year-old frogs, which suggests parasite-induced mortality. At POND B, the curve is flat, but the peak may have occurred at an earlier age outside the range of sizes sampled.

While the factors related to infection varied from one population to another, the genotype (*LL* or *RL*) was always involved. The selected GLMs were (genotype \* intensity of *R. bufonis*) at POND B, [condition + (genotype \* age)] at POND C, and [(genotype \* age)] at POND A. POND B population was the only one in which the 2 parasites influenced each other. However, the influence of the intensity of *R. bufonis* on that of *H. cylindracea* diverged dramatically according to genotype (Fig. 1). Whereas *LL* frogs carried higher numbers of *H. cylindracea* than *RL* frogs, the latter carried higher numbers of *R. bufonis*. Moreover, the intensity of both parasites was positively related in *LL* frogs whereas it was negatively related in *RL* frogs.

#### DISCUSSION

Despite significant variation among populations, our results clearly show that genotype (parental *versus* hybrid) strongly influences the intensity of parasites (genotype was involved in 4 out of 5 combinations of populations and parasite species). The other significant factors were body condition and age (involved in 3 combinations). The parasites were shown to influence each other in only 1 population (POND B). Nevertheless, the incidence of lung parasites in the two frog taxa studied varied greatly from one population to another. Indeed, the infection pressure by *H. cylindracea* was higher in POND B than in the other ponds. Concomitant variation of population structure and survival suggests that local ecological conditions influenced both infection frequency and fitness. However, the descriptive nature of this study prevents us from firmly identifying the causes of these differences. Both the variation in the density of intermediate hosts in the pressures of other pathogens, and the difference in

predation intensity on frogs between ponds (e.g. due to variation in predator density) could explain the observed patterns.

The parasite *H. cylindracea* proved to be the most effective for discriminating the responses of each genotype since the only significant result with *R. bufonis* was a similar influence on condition in both taxa at POND A (*R. bufonis* was related to a depression of condition with age). Despite variation in the responses from one pond to another, the present results suggest that *LL* frogs were more susceptible to *H. cylindracea* than *RL* frogs. This statement is supported by several results. In POND A, the intensity of *H. cylindracea* in *LL* frogs decreased with age, although it increased in *RL* frogs, suggesting a lower deleterious effect in this taxon. Indeed, we suppose that the survival of heavily infested frogs was lower in *LL* frogs than in *RL* frogs, thus explaining the negative relationship between age and intensity. Moreover, the body condition of *RL* frogs in this pond did not decrease, despite higher parasite intensity, whereas body condition of *LL* frogs decreased with age, although their parasite intensity was lower. In POND C, the intensity of *H. cylindracea* was negatively related to body condition in both taxa. Yet, the relationship was stronger in *LL* frogs than in *RL* frogs. However, this result has to be considered with some degree of caution because of the low number of *LL* frogs sampled in this pond. This relative scarcity of *LL* frogs could also be the result of the impact of parasites. Finally, the positive relationship between the intensity of each of the parasites in *LL* frogs in POND B suggests that the defence of this taxon is lowered by the presence of another parasite, although this was observed in *RL* frogs. Other factors can also contribute to the relationship between age and intensity, such as acquired immunity or dispersal of infected frogs.

The correlative nature of the results indeed prevents us from firmly concluding that the parasites studied have a direct impact on frog condition and frog survival. Unfortunately, very few data are available in the literature on the impact of *H. cylindracea*



infection on amphibians. The first host of this fluke is usually a snail of the genus *Lymnaea* (Goumghar *et al.* 2000). The cercariae metamorphose into metacercariae in the mouth of the frog where they stay for 48 h before metamorphosing into adults that migrate into the lungs where they feed on blood (Grabda-Kazubska and Moczo, 1981). In contrast, the deleterious impact of *R. bufonis* has been studied in toadlets (Goater, 1992; Goater and Ward, 1992). The impact on growth is positively related to the intensity of infection, and this effect is at least partially mediated by a depression of foraging propensity (anorexia effect). Unlike *H. cylindracea*, the biological cycle of *R. bufonis* can be realised with only 1 host (Baker, 1979). *Rhabdias bufonis* is a protandrous hermaphrodite, which undergoes heterogonic development. Embryonated eggs produced by females in the lungs are carried out by the bronchial escalator, swallowed, pass through the intestinal tract and are then voided in the faeces. These eggs hatch in the environment and the larvae produce a free-living generation of adults. This heterogonic development in the external environment is extremely rapid. Females produce a few large eggs, which develop to infective third-stage larvae in her uterus. The larvae then consume her organs and escape through her body cuticle in a process known as matricidal endotoky. These infective larvae infect a frog through skin penetration. In general, parasites with direct life-cycles, such as *R. bufonis*, are unlikely to manipulate the behaviour of their host to favour transmission to a downstream host. However, Baker (1979) demonstrated that paratenic hosts might be involved in the life-cycles of species of *Rhabdias*. The deleterious effects of infection are thus probably directly due to the alteration of lung functioning and blood consumption. Because the foraging behaviour of *H. cylindracea* is similar to that of *R. bufonis*, we suppose that the impact of this fluke is also directly due to physiological alteration resulting from damage to lungs and consumption of blood. Nevertheless, we cannot reject the hypothesis of age-dependent immune competence for explaining age-dependent parasitic infection (Cooper and Hildemann, 1965; Pickel *et al.* 1981; Ujvari and Madsen, 2006). It may be suspected that the two taxa of frog differ in the risk of exposure to parasites because of difference in habitat use (Hellriegel and Reyer, 2000). However, the available literature does not support such a proposal, neither in juveniles (Tietje and Reyer, 2004) nor in adults (Holenweg-Peter, 2001).

In the present study, we decided to focus on the infection of female frogs because of their crucial role in population dynamics. However, parasitic infection in both males and juveniles should also influence host population dynamics. Despite the low probability of male shortage, because of polygynous mating system (Lengagne *et al.* 2006), the males could act as reservoirs for parasites. However, one could

probably expect an important impact of parasites on juvenile survival because of suspected age-dependence of immunity efficiency. These questions should be investigated.

The variability of the responses suggests that other factors than the studied parasites influence frog condition and survival. The lung parasites may act simultaneously as determinants and indicators of a more global host-parasite-environment system. Water frogs can host a large community of parasites that inhabit their blood (Barta *et al.* 1989), urine bladder, digestive tract or kidneys (Tschermer, 1966; Peters, 1977). There is insufficient knowledge of the respective pathogenic power of each of them to define the assemblage that would be the more virulent and could strongly influence population dynamics. Moreover, the respective density of each pathogen can vary according to local conditions such as the density of intermediate hosts. On the other hand, variation in frog densities between ponds could also contribute to variation in infection probability and consequently in parasite densities. Nevertheless, we are confident in our result because of the great similarity of the studied ponds because traditional fishponds in the Dombes region have the same geomorphology, the same origin, the same environment and the same management.

With regard to the competing hypotheses regarding the sensitivity of the hybrids to parental parasites, the present results support the hybrid resistance hypothesis (due to an heterosis effect) than to the hybrid susceptibility hypothesis (breaking up of gene co-adaptation). Indeed, hybrid *RL* frogs did not suffer from higher parasitic intensity than the parental species that co-occurred with them in the same habitats. Parasite intensity in these hybrids appeared to be slightly lower than in the parental frogs. However, the theoretical implications of these results have to be interpreted with some caution because hybridogenetic *RL* frogs are not strictly equivalent to F1 hybrids. Water frog hybrid populations are composed of several lineages, arising from a more or less ancient primary hybridization between *R. ridibunda* and *R. lessonae*. The most ancient lineages probably originated during the last glacial events (Hotz *et al.* 1997). Although primary hybridization probably occurred repeatedly, only few hemiclone lineages are found in natural populations of *RL* frogs (Colon, 2004). This low hemiclone diversity may result from natural selection by both intrinsic biological factors (compatibility with the *lessonae* genome) and ecological ones (adaptation to habitats usually occupied by *R. lessonae* are necessary for achieving sexual parasitism). Parasite assemblages are probably an important component of these ecological selective pressures.

Because of its descriptive character, the present study does not allow us to firmly establish causative links between the variables measured. Only

experimental infections would make it possible to rigorously test the heterosis hypothesis. However, conceiving experimental approaches requires a basic knowledge of the relationships that have to be detected in the wild. The present study thus provides several new insights into the relationship between heterozygosity and parasite infection and, more globally, into the evolution of hybrid-parasite systems. Further studies should address the question regarding the influence of sex on parasitaemia, since sex is another important source of variability in natural populations.

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