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One day is enough: rapid and specific host–parasite interactions in a stickleback-trematode system

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Red Queen models of host–parasite coevolution are based on genotype by genotype host–parasite interactions. Such interactions require a genotype specific host defence and, simultaneously, a genotype specific parasite infectivity. Specificity is defined here as defence or infection ability successful against only a subset of genotypes of the same species. A specific defence depends on detectable genotypic variation on the parasite side and on a host defence mechanism that differentiates between parasite genotypes. In vertebrates, the MHC-based adaptive immune system can provide such a defence mechanism, but it needs at least several days to get fully mounted. In contrast, the innate immune system is immediately ready. The trematode parasite species used here reaches the immunologically protected eye lens of its three-spined stickleback (*Gasterosteus aculeatus*) host within 24 h. Thus, it disappears too fast for the fully mounted MHC-based adaptive immune system. In a complete cross-infection experiment using five fish-families and five parasite-clones, we found for the first time fish-family by parasite-clone interactions in vertebrates, although the parasite was only exposed to the immune system for maximally one day. Such interactions require a fast genotype specific defence, suggesting the importance of other defence mechanisms than the too slow, fully mounted adaptive immune system in vertebrates.

Keywords: innate immunity; adaptive immunity; fast and specific defence; *Diplostomum pseudospathaceum*; *Gasterosteus aculeatus*

1. INTRODUCTION

Host–parasite coevolutionary theory and Red Queen dynamics have often been considered the major cause for the maintenance of sexual reproduction and genetic diversity (Hamilton 1980; Paterson 2005). A main assumption of these models is the presence of genotype by genotype host–parasite interactions (Carius *et al.* 2001). Hosts show resistance specific against a particular set of parasite genotypes and simultaneously parasites show infectivity specific to a particular set of host genotypes (Schmid-Hempel & Ebert 2003). Throughout this paper, specificity is defined as a defence or infection ability that is successful against some genotypes, but not against other genotypes of the same species (Schmid-Hempel & Ebert 2003).

For a genotype specific defence two requirements have to be met. First, variation in parasite genotypes must be detectable, for example, in the parasite surface structures (antigens) used by the host immune system for recognition. Second, the defence is only mounted against a restricted subset of genotypes defined by specific recognizable antigens. As a well-studied case in point, all vertebrates possess such a highly variable defence mechanisms, the MHC-based adaptive immune system (Frank 2002). The induction of the adaptive immune system needs at least 3–5 days and up to several weeks in poikilothermic bony fishes (Magnadóttir 2006). This delay is partly due to the time needed for the lymphocyte proliferation, a necessary step to build up an effective adaptive immune defence (Magnadóttir 2006). In contrast, the innate immune system is immediately ready. As many innate defence mechanisms rely on the recognition of structures shared by large groups of parasites, they are regarded as rather unspecific, that is the defence does not distinguish between different parasite genotypes (Kurtz 2005). However, the innate immune system also possesses recognition mechanisms able to differentiate between different antigens, involving for example, natural antibodies, Toll and lectin-like receptors (Tchernychev *et al.* 1997; Kurtz 2005; for fish innate immune system see Magnadóttir 2006). Note that a genotype specific host defence alone will not produce, but is a prerequisite, for genotype by genotype host–parasite interactions.

Genotype by genotype (or isolate) interactions have often been found in plants (e.g. Karban 1989) and invertebrates (e.g. Carius *et al.* 2001), but only rarely in vertebrates (Paterson 2005; but see Benjamin *et al.* 1986 for an example). This evidence for genotype by genotype interactions was only found six or more days after infection. However, many parasite species can escape within a few hours from the host immune system by means of evasion strategies (Chappell *et al.* 1994). A specific defence relying on mechanisms of the adaptive immune system that need several days to become fully mounted are to slow to influence such host–parasite interactions.

To study fast host–parasite interactions in vertebrates, we used the three-spined stickleback fish (*Gasterosteus aculeatus*) and the trematode parasite *Diplostomum pseudospathaceum* as our model system. The parasite penetrates the skin of the fish and migrates within 24 h through the body into the eye lens, where it is protected from the immune system (Chappell *et al.* 1994). We used five different parasite genotypes, each composed of genetically identical individuals (parasite-clone). As a surrogate for five fish genotypes, we used five fish-families, each composed of full-siblings (fish-family). In a complete cross-infection experiment, fishes of each family were infected in single-clone infections with each parasite clone. As such, our assessment of genotype by genotype interactions is conservative because on the fish side, individuals within a family share, on average, only 50% of their genetic background. In vertebrates, genotype by genotype host–parasite interactions present within 24 h and thus not relying on

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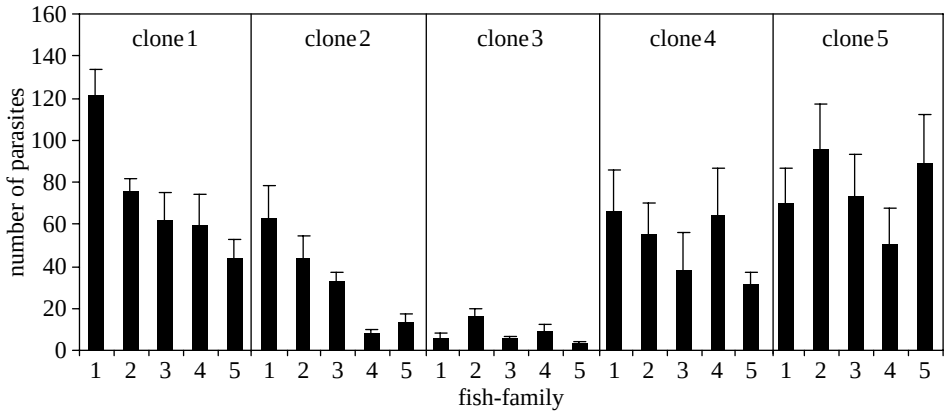

Figure 1. Rapid and specific interactions between fish-families and parasite-clones. Bars show mean number of parasites (±s.e.) per fish in the 25 fish-family parasite-clone combinations.

the fully mounted mechanisms of the adaptive immune system, have to the best of our knowledge not been demonstrated yet.

2. MATERIAL AND METHODS

(a) *Study system*

The trematode parasite *D. pseudospathaceum*, displays a complex life cycle typical for digenean trematode species. Sexual reproduction takes place in the gut of fish-eating birds. Parasite eggs are shed into the water and hatching larvae infect water snails, where asexual reproduction occurs. Snails release free-swimming larvae, which infect fishes, such as the three-spined stickleback (*G. aculeatus*).

We established five clonal lines of the parasite *D. pseudospathaceum*. We isolated parasite eggs from faeces collected from herring gulls (*Larus argentatus*) living in a large colony on an island of an interconnected lake system in northern Germany. We added single hatched larvae to single water snails (*Lymnaea stagnalis*) from a large laboratory breed originating from the same lake system. Successful parasites reproduced asexually within the snails and after two months, snails started to release stages ready to infect fishes. All parasites were released from one snail belonged to one clone. We included only clones originating from faeces of different gulls.

Parasite clones were identified morphologically as *D. pseudospathaceum* (Niewiadomska & Kiseliene 1994). For taxonomic verification, we used ribosomal DNA-ITS sequences (Internal Transcribed Spacer) to verify that the clones of *D. pseudospathaceum* used belong to the same species. Non-coding ITS sequences are widely used for phylogenetic analysis due to the within-species homogeneity and between species divergence (White *et al.* 1990).

To establish fish-families, we caught wild fishes from the same lake system, from which the parasite and the snails originated. We produced 10 unrelated F1 fish-families. From each F1 family, we used only one fish. We formed five pairs and produced five F2 families (for details on crossing procedure and rearing conditions see Rauch *et al.* 2006).

(b) *Parasite infection design*

In single-clone infections, we infected fishes of each of five fish-families with each of five parasite clones, resulting in a complete cross-infection experiment with 25 fish-family parasite-clone combinations. We used five fishes from each family for each clone (except for one fish-family, where only four fishes were used due to small offspring number in that family). We placed the fishes singly in 1 l aquaria. To each fish, we added 150 parasites of the respective clone into the water not later than 12 h after the parasites emerged from the snails. We randomly distributed the aquaria on tables in a climate chamber (18 °C, 16 h light). We allowed the parasites to grow for one week in order to minimize counting mistakes, as the bigger stages are more easily detectable. We then killed the fishes with an excess of methane sulphonate (MS 222), dissected and isolated the eye lens and counted the parasites within the undamaged eye lens.

(c) *Data analysis*

We analysed infection data with a fully crossed two-way ANOVA with number of parasite individuals per fish as response variable and parasite-clone and fish-family included as random effects. The interaction effect was measured between the two random effects parasite-clone and fish-family. Parasite counts were log transformed to restore normal distribution (Kolmogorov–Smirnov test) and homogeneity of variances (Cochran test).

Table 1. Effects of fish-family and parasite-clone on the number of parasites per fish and the interaction between fish-family and parasite-clone, analysed with a two-way ANOVA.

source of variation	d.f.	*F*-value	*p*
fish-family	4.16	3.88	0.0218
parasite-clone	4.16	24.57	<0.0001
fish-family×parasite-clone interaction	16.82	1.97	0.0249

(d) *Long-term survival*

Although parasites are supposed to be immunologically protected within the eye lens (Chappell *et al.* 1994), we wanted to verify that no unknown host defence mechanism reduces parasite number within the eye lens over time, nor that parasite competition or nutrient shortage would decrease parasite numbers. To test for such a decrease, we infected four fishes of each of the five families, where each fish was exposed to 150 parasites (30 parasites of each clone). We estimated numbers of parasites for the same individuals one week and nine weeks after infection. An estimation of parasite numbers in living fishes is possible by visual examination of the eye lens through the pupil.

3. RESULTS

We identified a significant fish-family by parasite-clone interaction (figure 1; table 1). No parasite-clone performed best in all fish-families and no fish-family had the highest resistance against all clones. Clone identity had an effect on the number of parasites per fish (table 1). Also, fish-family influenced number of parasites (table 1).

We did not find a significant decrease in number of parasites from the first to the ninth week after infection (paired *t*-test: $t_9 = 0.55$; $p = 0.5960$). This indicates that our findings are not influenced by later acting defence mechanisms. Ten fishes died one day after the first check, which was probably due to handling stress during the parasite screen.

Sequence analysis of the ITS of the ribosomal DNA genes revealed that a 566 base-pair fragment of the first spacer and a 214 base-pair fragment of the second spacer were 100% identical for all five experimental *D. pseudospathaceum* clones (three replicates per clone). An NCBI BLASTn search using the more frequently analysed first spacer fragment

showed that there were zero nucleotide differences compared to a sequence from *D. pseudospathaceum* (GenBank accession number: AF419273.1), but four changes compared to the next closest match (*Diplostomum indistinctum*: AY123043.1). Thus, there were no hidden *Diplostomum* species that influenced our experimental results.

4. DISCUSSION

Genotype by genotype host–parasite interactions are one of the main prerequisites for Red Queen dynamics and coevolution theory (Hamilton 1980). This study found for the first time fish-family by parasite-clone interactions in vertebrates, although the parasite was only exposed to the immune system for maximally one day. Such interactions require a genotype specific defence and simultaneously a fish-family specific parasite infectivity. The fully mounted defence mechanisms of the adaptive immune system are too slow to influence such host–parasite interactions. A fast and specific defence is particularly important for genotype by genotype host–parasite interactions in those parasite species that rapidly escape from the recognition of the host, for example, by hiding in the eye lens as in the parasite species used here. The postulated defence mechanism is either built up within a few hours or it is constitutively ready to use. Our findings add to the recent debate on the complexity of mechanisms involved in specific defence other than the well-studied mechanisms of the adaptive immune system (Kurtz 2005; Little *et al.* 2005).

The molecular mechanisms involved in the specific defence proposed here are as yet unknown. Possible candidates include lectin-like receptors, which recognize surface sugar compounds on the parasite surface and are present in several fish species (reviewed in Magnadóttir 2006). Surface sugar composition on different parasite clones may differ, leading to different binding affinities by the lectin-like receptors to different parasite genotypes, which may reciprocally vary with host genotype. The difference in binding affinity may then determine the strength of the defence (Roitt *et al.* 1989). Such a defence would be instantly ready and could differentiate between different genotypes.

Other possible candidates for the underlying molecular mechanism are natural antibodies, which are widespread in fishes (reviewed in Magnadóttir 2006). Natural antibody reaction can differentiate between different parasite antigen (Tchernychev *et al.* 1997). Natural antibodies are produced without any antigen stimulation and are therefore immediately ready.

Although the underling molecular mechanism remains to be investigated, experimental evidence at the phenomenological level is an essential first step, before uncovering the physiological mechanism (Kurtz 2005). This guideline was successful in exploring for example the adaptive immune system (Little *et al.* 2005). In this study, we found fish-family by parasite-clone interactions, even if the parasite was only exposed to the immune system for one day, long before the adaptive immune system is ready.

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