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Differences in initial and acquired resistance to *Ichthyophthirius multifiliis* between populations of rainbowfish

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Wild-caught rainbowfish *Melanotaenia spp*. originating from three isolated populations were infected with a quantified dosage of parasites *Ichthyophthirius multifiliis* in a controlled environment. The *Melanotaenia eachamensis* from Dirran Creek were much more susceptible to ichthyophthiriasis than were *M. splendida* from the Lake Tinaroo or Bluewater Creek populations. When the highly susceptible Dirran Creek rainbowfish were crossed with rainbowfish from a fourth population, Lake Eacham *M. eachamensis*, they produced hybrids with significantly higher resistance than pure-bred Dirran Creek, but not higher than pure-bred Lake Eacham fish. Hence, intraspecific hybridization increased resistance to *I. multifiliis* infection in *M. eachamensis*. Hosts from all three populations were much less susceptible to infection on their second exposure to the parasite. However, the Bluewater Creek population was better able to acquire immunity to *I. multifiliis* than either the Dirran Creek or Lake Tinaroo populations. It is tentatively suggested that there may be a link between the heterozygosity of populations of rainbowfish and their initial ability to resist infection by *Ichthyophthirius multifiliis*.

Key words: acquired; parasite resistance; *Melanotaeniidae*; *Ichthyophthirius multifiliis*.

INTRODUCTION

Different fish species, and different populations or strains of a single species, often show significant differences in their ability to resist disease (Hines *et al*., 1974). As early as 1947, Butcher observed that rainbow trout *Oncorhynchus mykiss* (Walbaum) were far more susceptible to infection by *Ichthyophthirius multifiliis* (Fouquet) than were brown trout *Salmo trutta* L. Later, in a controlled experiment, Clayton & Price (1992) demonstrated that susceptibility to ichthyophthiriasis varied between different strains of a poeciliid species, *Xiphophorus maculatus* (Günther).

Such variation in disease-resistance between host species, or between populations, may be due to the health status of the individuals, which may be influenced largely by environmental factors (Hoffmann & Parsons, 1991). For example, resistance against internal parasites of the digestive tract can be modified by the types of foods which are eaten, or when and where the host feeds (Freeland, 1983). However, an increasing number of authors are providing evidence that disease-resistance is, at least partly, under genetic control (Gordon, 1953; Hines *et al*., 1974; Clayton & Price, 1994; Price & Clayton, 1999).

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Unfortunately, many reports of variation in disease-resistance, especially among fish strains or species, have been inferred from naturally observed parasite burdens in aquaculture or after epizootics in the wild, rather than explicit studies of resistance. In the absence of standardized experimental exposure to parasites, this leaves open the possibility that differences between species or strains in parasite burden are due to differences in exposure to parasites rather than difference in resistance, let alone differences in the genetic makeup of hosts (Owens & Wilson, 1999). Unfortunately, many parasites cohabiting naturally on wild animals are difficult to culture in the laboratory.

An excellent parasite for use in laboratory studies investigating parasite–host relationships is *I. multifiliis* because a direct, quantifiable measure can be obtained non-destructively by counting the individual parasites. Also, important from an ethical view, infected fish can be disinfected quickly. Commonly known as ' Ich ', the causative agent of the white spot disease (ichthyophthiriasis), this protozoan can infect most, if not all, freshwater fish (Ventura & Paperna, 1985) and at least one species of amphibian (Gleeson, 1999). It has a direct life cycle with a dispersive, infective theront stage and a mature trophozoite, which lives immediately under the epidermis of the host (McCallum, 1982).

In recent years, there have been significiant advances in elucidating the mechanisms of host defence against *I. multifiliis* (Dickerson & Clark, 1998). Host resistance to the parasite involves initial (non-specific) resistance and acquired (adaptive) resistance. Initial (non-specific) resistance to *I. multifiliis* consists of physical and chemical mechanisms for resisting infection, including non-specific cytotoxic cells (Evans *et al.*, 1998). The nature of the epidermis may also help to prevent infection. For example, Price & Clayton (1999) found that fully scaled carp *Cyprinus carpio* L., were more resistant to *I. multifiliis* than incompletely scaled breeds of carp.

Acquired (or adaptive) resistance utilizes the immune defence system of an organism. After a fish has been exposed to *I. multifiliis*, serum and mucous antibodies are synthesized to attack the parasite (Clark *et al.*, 1988). These specific antibodies are resynthesized if the organism is exposed subsequently to the same parasite (Clark *et al.*, 1988). This mechanism results in complete, or partial, resistance to infection, otherwise known as immunity. Recent studies have suggested that rather than killing the infective stages of *I. multifiliis* the parasites may be forced to exit the fish prematurely in response to antibody binding (Wahli & Matthews, 1999).

The objectives of this study were to investigate possible differences between rainbowfish populations in initial and acquired resistance to *I. multifiliis*, and to determine whether intraspecific hybridization can increase resistance to infection. As it is extremely impractical to look at acquired immunity in the field, a controlled laboratory study was chosen. The experiments were designed to minimize all sources of variation apart from genetic factors, including variation in exposure to the parasite.

METHODS

SOURCE OF FISH

Rainbowfish from four populations in north Queensland, Australia were used: Dirran Creek (*Melanotaenia eachamensis* Allen & Cross; 17°28′ S; 145°33′ E), Tinaroo

Dam's Fong on Bay (*Melanotaenia splendida splendida* Peters; 17°10′ S; 145°35′ E), Lake Eacham (*Melanotaenia eachamensis*; 17°17′ S; 145°38′ E), and Bluewater creek (*M. splendida splendida*; 19°11′ S; 146°33′ E).

These populations are isolated, but closely related (see Zhu *et al*., 1998). *M. eachamensis* was thought to be a recent derivative of *M. splendida* (Allen & Cross, 1982; Merrick & Schmida, 1984). However, whether or not *M. eachamensis* and *M. spendida* are actually separate species has also been doubted over the years (Wager & Jackson, 1993). The two species hybridize readily in captivity to produce viable offspring (Allen & Cross, 1982). A recent survey by Zhu *et al*. (1998) revealed that Lake Eacham and Dirran Creek rainbowfish (both classified *M. eachamensis*) contained pure *eachamensis* mtDNA halotypes. In contrast, Lake Tinaroo rainbowfish (formally classified as *M. splendida*) contained a mixture of *eachamensis* and *splendida* mtDNA halotypes (Zhu *et al*., 1998).

The rainbowfish from Dirran Creek, Lake Tinaroo and Bluewater Creek were captured from the wild using nets, transported to the University of Queensland and allowed to acclimatize at a constant temperature of 22° C for at least a month prior to experimentation. Ages of these fish were not known because they were wild-caught.

Hybrids of Dirran Creek × Lake Eacham were bred specifically by crossing wild-caught Dirran Creek fish with captive Lake Eacham stock. In addition, pure strains of Lake Eacham and Dirran Creek stock were also bred to give directly comparable reference populations. All progeny of the crosses were the same age, and thus of similar sizes. The pure Dirran Creek strains were the result of crosses between randomly selected wild-caught fish. The Lake Eacham rainbowfish used to breed the experimental fish were taken from captive stock because no Lake Eacham rainbowfish remain in the wild. None of the F1 progeny had developed icthyophthiriasis prior to experimentation.

SOURCE OF PARASITE

In order to have a readily available source of *I. multifiliis* parasites, a parasite culture was set up. The materials and methods used to maintain the culture were modified from McCallum (1982) and Clayton & Price (1988). Fish already infected with *I. multifiliis* were selected from a single tank in a retail aquarium shop. Serial transmission between a variety of aquarium fish species, including black mollies *Poecilia latipinna* Lesueur and rainbowfish maintained the strain of *I. multifiliis*. In order to create uniform, high levels of parasites in culture, large numbers of healthy fish were placed in the same tank with *c*. 10 infected fish. After 3 days, the original 10 fish were removed and treated with a white spot medication (Rapid White Spot Remedy, Aristopet).

Six or 7 days after the parasite culture was commenced, the newly infected fish were placed in 400 ml water for 45 min so that the agitated movements of the hosts would cause the mature trophozoites to dislodge (Clayton & Price, 1988). Once the trophozoites had been harvested, the hosts were used as heavily infected fish in the parasite culture methods above, so that the strain was maintained.

The beaker of water containing mature trophozoites was incubated for 18–20 h at 22° C (initial trials established that at this temperature, theronts hatched within 18–20 h). During incubation, the trophozoites undergo rapid mitotic division to form theronts (highly mobile, host-seeking stage of the parasite). In order to determine the concentration of theronts present in the suspension, the mean concentration in nine repeated counts using 0·1-ml samples was calculated. This was achieved by stirring the suspension briefly in order to homogenize the mixture, drawing the sample into the pipette, and placing the sample on a Sedgewick Rafter (550 ml, Graticules Limited). The number of theronts present under 100 × magnification was counted after immobilization of theronts with a small amount of formalin.

INFECTION

During the experimental exposure of hosts, each individual rainbowfish was placed in an opaque, plastic container containing 400 ml of aged water at 22° C. *I. multifiliis* is thought to have weak long-range chemotaxis (Lom & Cherkasovova, 1974), so a small volume of water was used to maximize the chance of the parasites contacting the fish. Then, each experimental rainbowfish was exposed to *c*. 2000 theronts by adding the

appropriate volume of contaminated water. All fish were infected at the same time, randomly. The containers were covered with a sheet of polystyrene foam to prevent the fish from jumping out. Each experimental exposure was terminated on the fourth day, by which time any trophozoites were large enough to be counted readily. So that the parasites would have maximum opportunity to infect the experimental fish, the water in the containers was not changed during the infection period.

In order to determine the degree of parasitic infection, individual fish were anaesthetized lightly using ethyl p-aminobenzoate made up as a primary solution of 1 g 100 ml^{-1} 80% ethanol (Clayton & Price, 1988). The anaesthetic was added to the water slowly until the fish lost motor activity but not respiratory activity. The fish were examined under a stereomicroscope using a cold light source while in a petri dish of water. The body surface area, including fin area, of each fish was determined using the computer program, NIH Image (public domain software), on an Apple Macintosh computer, connected to a video camera. The body surface of the rainbowfish was divided into regions and the number of parasites present in each region recorded separately (Clayton & Price, 1988). This method was designed to minimize error incurred through counting a single parasite twice.

EXPERIMENTAL PROTOCOL

Two separate experiments were conducted in order to test whether rainbowfish populations differed in either their initial or acquired ability to resist parasitic infection by *I. multifiliis*. Initial resistance was measured by exposing naive individuals (20 Dirran Creek, 20 Bluewater Creek (group 1—see later) and 20 Lake Tinaroo) to *c*. 2000 theronts. Counting the number of parasites on the fish's skin 4 days later assessed the parasite load of each individual. Then, each fish was treated immediately with medication in order to disinfect it. Fish were kept in their individual containers between the initial and subsequent exposures so that the identity of each fish would be known. During this time, periodic water changes were made.

The extent of the acquired resistance was indexed by re-infecting these fish with 2000 theronts per fish 2 weeks later. The sample size was reduced to 17 for Lake Tinaroo due to deaths. Acquired resistance was calculated as the ratio of the infection level (parasite burden) after the second exposure, to infection level after the first exposure. Of course, this measure of acquired resistance relies on the parasites themselves being equally infective on first and second exposure. To check that this assumption was met, an additional group of naïve rainbowfish (group 2) from the Bluewater Creek population was infected also using the second batch of theronts. If these two batches of theronts were equally infective, there should be no significant differences in the mean parasite burden of groups 1 and 2.

In a third experiment, rainbowfish from the Dirran Creek population were hybridized with rainbowfish from captive Lake Eacham stock. Each of 20 hybrid individuals, and 20 of each of the parental populations, was infected with a low parasite dosage of 150 theronts per fish. A lower dose of theronts was used here than in the first experiment because these hybrid fish were exposed when at a smaller size. The resultant parasite burden was assessed four days after infection.

STATISTICS

An analysis of covariance (ANCOVA) on square-root-transformed data, with fish body surface area as a covariate, was used to determine whether there were any interpopulation differences in parasite burdens of hosts on first and second exposure to *I. multifiliis*. A least squares means test, with a Tukey–Kramer correction for multiple comparisons, was used to determine which populations differed from each other. A *t*-test was used to determine whether fish from the Bluewater Creek population that had been exposed to *I. multifiliis* had significantly lower parasite burdens than Bluewater Creek fish not previously infected (controls).

In order to determine if immunity was acquired, ratios of parasite burden after the first infection: parasite burden after second infection of each individual fish were calculated. Thus, a ratio of one indicated no acquired resistance, while a ratio greater than one

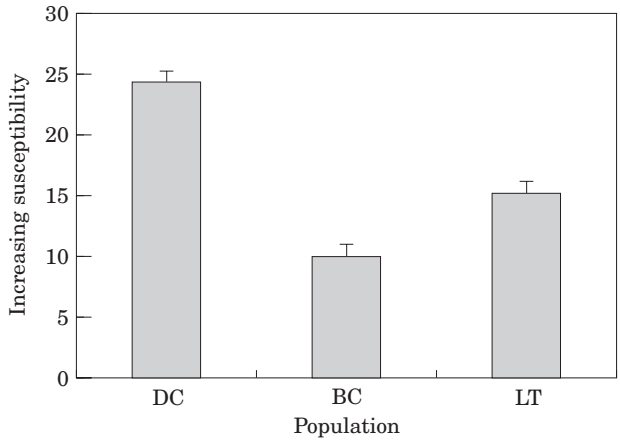

FIG. 1. Differences in susceptibility to *Ichthyophthirius multifiliis* between Dirran Creek *M. eachamensis* (DC), Bluewater Creek *M. splendida* (BC) and Lake Tinaroo *M. splendida* (LT). The scale of increasing susceptibility is based on least squares mean values for square root transformed data and compares populations adjusted for size. Error bars indicate the S.D. for each data point.

indicated acquired resistance. A non-parametric Kruskal–Wallis test was used to determine whether the populations differed in ability to acquire immunity to ichthyophthiriasis. An ANCOVA was used to determine whether the degree of resistance to infection depended on the intensity of the initial infection.

A one-way analysis of variance (ANOVA) on square-root-transformed data was used to determine whether there was significant variation among the purebred and hybrid progeny with respect to initial resistance to ichthyophthiriasis. A preliminary ANCOVA demonstrated that neither fish body size nor sex had a significant effect on the population effect, and these were removed subsequently from the final analysis. A least squares means test, with a Tukey-Kramer adjustment was used to determine which populations differed in resistance to ichthyophthiriasis. All statistical analysis was performed using the SAS package (Version 6.12). Two-tailed probabilities are reported throughout.

RESULTS

There was a highly significant difference between all three populations in their initial resistance to ichthyophthiriasis ($F=77{\cdot}12$, d.f.$=2,55$, $P=0{\cdot}0001$; Fig. 1). The Dirran Creek fish had significantly more parasites than fish from both of the other populations (LSM test, $P=0{\cdot}0001$). However, Lake Tinaroo fish also had significantly more parasites than Bluewater Creek fish (LSM test, $P=0{\cdot}0033$). A relationship between fish body surface area and parasite load was detected, whereby fish with large body surface areas had smaller parasite loads ($F=6{\cdot}31$, d.f.$=2,55$, $P=0{\cdot}014$). This relationship had equal slope for all three populations ($F=2{\cdot}28$, d.f.$=2,53$, $P=0{\cdot}125$). The sex of the fish did not appear to affect parasite load.

The parasite burdens after the second exposure were much lower than after the first exposure, with all populations having between five and eight times less parasites (Table I). These differences in parasite burden between first and second exposure must be due to acquired resistance because exposure of naïve Bluewater Creek control groups showed there was no significant difference in the infectivity

TABLE I. Differences in resistance to ichthyopthiriasis after first and second exposure to *Ichthyophthirius multifiliis*. Mean parasite values were obtained by back-transforming least squares mean values for square root-transformed data

Population	First exposure (mean no. parasites fish^{-1})	Second exposure (mean no. parasites fish^{-1})	Ability to acquire resistance (ratio of 1st : 2nd exposure)
Dirran Creek (*M. eachamensis*)	592·259	108·547	5·456
Lake Tinaroo (*M. splendida*)	226·176	40·861	5·535
Bluewater (*M. splendida*)	98·377	9·112	10·796

of the theronts in the second batch compared with the first batch (t=0·73, d.f.=34, P=0·23).

The populations differed significantly with respect to parasite burden after the second exposure (F=20·00, d.f.=3,66, P=0·001). The parasite load after the second exposure was significantly lower in the Bluewater Creek than the Dirran Creek population (LSM test, P=0·0001), and significantly lower in the Lake Tinaroo than the Dirran Creek populations (LSM test, P=0·017). However, there was no significant difference in parasite loads between the Bluewater population and the Lake Tinaroo population (LSM test, P=0·1142).

Additionally, there was a significant difference between the population in their ability to acquire resistance (χ^2=6·838, d.f.=2, P=0·03, Kruskal-Wallis test comparing populations for the ratio of parasite burden in the first exposure with that in the second exposure). The fish from the Bluewater Creek population acquired greater immunity to ichthyophthiriasis than the Dirran Creek population (χ^2=7·53, d.f.=1, P=0·0061). No significant difference in ability to acquire resistance could be detected between the Dirran Creek and Lake Tinaroo populations (χ^2=1·49, d.f.=1, P=0·2228), or between the Lake Tinaroo and Bluewater Creek populations (χ^2=1·00, d.f.=1, P=0·3091). The degree of resistance did not depend on the intensity of the initial infection (F=0·007, d.f.=1,48, P=0·79).

The intraspecific hybridization resulted in significant differences in susceptibility to infection between the crosses (F=10·79, d.f.=2,26, P=0·0001; Fig. 2). The Dirran Creek × Dirran Creek progeny had a significantly higher parasite load than the new hybrid progeny (LSM test, P=0·0004) and the Lake Eacham × Lake Eacham progeny (LSM test, P=0·0113). The parasite burdens resulting from this experiment (Fig. 2) are lower than those for the initial exposure in the initial experiment (Fig. 1) because, as explained in the methods, a lower dose of parasites was used during this experiment.

DISCUSSION

Highly significant differences in initial resistance to parasite infection existed between the genetically distinct populations of fish. This is in accordance with Clayton & Price's (1992) finding that resistance to ichthyophthiriasis varies among domestic strains of *Xiphophorus maculatus*. However, the current study

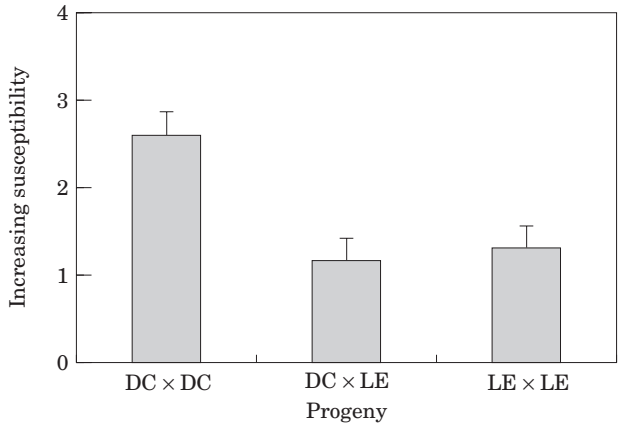

FIG. 2. Differences in susceptibility to *Ichthyophthirius multifiliis* between pure strains of Dirran Creek (DC × DC) and Lake Eacham (LE × LE) *M. eachamensis* and the progeny of an intraspecific hybridization of the two populations (DC × LE). The scale of increasing resistance is based on least squares means for square root-transformed data. Error bars indicate the S.D. for each data point.

appears to be the first report of interpopulational differences in resistance between *natural* fish populations for ichthyophthiriasis. Furthermore, the ability to acquire resistance to ichthyophthiriasis also varied significantly between natural populations of rainbowfish.

The reason why the rainbowfish populations differed in initial and acquired ability to resist ichthyophthiriasis is not clear. One possible explanation is that the effects observed are genotype specific. That is, the Dirran Creek population of rainbowfish used in the experiment may have been unusually susceptible, or the Lake Tinaroo and Bluewater Creek populations may have been unusually resistant to the parasite in question.

Here, evidence is provided that intraspecific hybridization can increase parasite resistance in *M. eachamensis*. By experimentally hybridizing the particularly susceptible Dirran Creek population with another, more resistant population, a new hybrid population was produced that shared many of the attributes of the original populations. The production of such a hybrid population led to a large increase in parasite resistance relative to the Dirran Creek population. Thus, this study added support to the notion that resistance to ichthyophthiriasis is under genetic control. In 1994, Clayton & Price provided evidence for interspecific heterosis in resistance to *I. multifiliis* under very similar, controlled conditions as used in the current study.

Genetic variation between individuals in resistance to disease may be under the control of major genes such as tumour development in xiphophorid hybrids, or several genes, acting in an additive manner (Price & Bone, 1985). Recently, Price & Clayton (1999) demonstrated that genotype-environment interactions influence the susceptibility of common carp to *I. multifiliis* infections.

One of the most controversial explanations for variation between strains or populations in disease-resistance is that resistance may be governed by the level of heterozygosity of individuals in a population (Allendorf & Leary, 1986). Recent published work based on infectious disease in natural populations

generally supports the assumption that more heterozygous individuals have a higher resistance to parasites (Ferguson & Drahushchak, 1990; Markowski *et al.*, 1990; Gulland *et al.*, 1993; Preleuthner *et al.*, 1995). Similarly, losses of heterozygosity in desert fish *Poeciliopsis* spp. have been associated with increased susceptibility to parasites (Vrijenhoek, 1996).

Another explanation for the differences in resistance between populations found in this study may be that the differences in susceptibility reflect the amount of genetic variation present in the population. The Dirran Creek, Lake Eacham and Lake Tinaroo populations were known to differ in their level of genetic heterozygosity [$0{\cdot}19 \pm 0{\cdot}11$ (S.E.); $0{\cdot}30 \pm 0{\cdot}12$; and $0{\cdot}68 \pm 0{\cdot}04$, respectively], as assessed by microsatellite loci markers at four loci (Zhu *et al.*, 1998). These estimates were assumed to be roughly representative of present fish samples.

Dirran Creek and Lake Tinaroo populations, which differed by a factor of approximately three in terms of heterozygosity, also differed approximately three-fold with respect to parasite burden following a standardized initial exposure. Although the Dirran Creek and Lake Tinaroo populations showed a pronounced ability to acquire resistance to the parasite, there was no significant difference in their ability to acquire such resistance. Unfortunately, there was no estimate of the heterozygosity of the F1 progeny of the Dirran Creek × Lake Eacham cross. However, the manipulated out-crossing, which can lead to increased heterozygosity as a consequence of increased allelic diversity, resulted in progeny more resistant than the purebred Dirran Creek progeny.

However, these experiments are not sufficient to demonstrate explicitly that parasite resistance is determined causally by genetic diversity. For example, the heterozygosity estimates were assessed using hypervariable microsatellite markers, and it seems very unlikely that these would be the exact genes involved in parasite resistance. In addition to this, the observed association between level of genetic diversity and parasite resistance could be purely coincidental because the high and low diversity populations were not replicated. Nevertheless these preliminary results provide a sound basis for further investigation. It would be interesting to investigate the effect of individual heterozygosity on the ability to resist parasites and to examine why there seems to be such a big difference between acquired and initial resistance.

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