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Source: Journal of Wildlife Diseases, 36(2) : 197-201

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-36.2.197

Journal of Wildlife Diseases, 36(2), 2000, pp. 197–204

PATHOGENICITY OF AVIAN MALARIA IN EXPERIMENTALLY-INFECTED HAWAII AMAKIHI

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ABSTRACT: The introduction of avian malaria (*Plasmodium relictum*) and mosquitoes (*Culex quinquefasciatus*) to the Hawaiian Islands (USA) is believed to have played a major role in the decline and extinction of native Hawaiian honeycreepers (Drepanidinae). This introduced disease is thought to be one of the primary factors limiting recovery of honeycreepers at elevations below 1,200 m where native forest habitats are still relatively intact. One of the few remaining species of honeycreepers with a wide elevational distribution is the Hawaii Amakihi (*Hemignathus virens*). We measured morbidity and mortality in experimentally-infected Hawaii Amakihi that were captured in a high elevation, xeric habitat that is above the current range of the mosquito vector. Mortality among amakihi exposed to a single infective mosquito bite was 65% (13/20). All infected birds had significant declines in food consumption and a corresponding loss in body weight over the 60 day course of the experiment. Gross and microscopic lesions in birds that succumbed to malaria included enlargement and discoloration of the spleen and liver and parasitemias as high as 50% of circulating erythrocytes. Mortality in experimentally-infected amakihi was similar to that observed in Apapane (*Himatione sanguinea*) and lower than that observed in Iiwi (*Vestiaria coccinea*) infected under similar conditions with the same parasite isolate. We conclude that the current elevational and geographic distribution of Hawaiian honeycreepers is determined by relative susceptibility to avian malaria.

Key words: Avian malaria, Drepanidinae, experimental infection, *Hemignathus virens*, honeycreeper, pathology, *Plasmodium relictum*.

INTRODUCTION

The accidental introduction of mosquitoes and avian malaria to the Hawaiian Islands (USA) has had devastating impacts on native forest bird communities and has led to profound landscape-level effects on the elevational and geographic distribution of highly susceptible native species, particularly endemic Hawaiian honeycreepers (Drepanidinae) (Warner, 1968; van Riper et al., 1986; Atkinson et al., 1995). While once abundant from sea level to tree line, honeycreepers are now found primarily in high elevation refugia on Kauai, Maui, and Hawaii where cooler temperatures limit the range of the primary vector of avian malaria in the islands, Culex *quinquefasciatus* (Scott et al., 1986; van Riper et al., 1986).

Of 16 species of honeycreepers from the main Hawaiian Islands that are still believed to be extant (Jacobi and Atkinson, 1995), Apapane (*Himatione sanguinea*), Oahu Amakihi (*Hemignathus chloris*), and Hawaii Amakihi (*Hemignathus virens*) are the only species with ranges that regularly extend to elevations lower than 600 m. Fragmented, low elevation populations of amakihi occur on the islands of Oahu and Hawaii in areas with abundant *Culex* mosquitoes (Scott et al., 1986; VanderWerf, 1997; C. T. Atkinson, unpubl. data). It is not clear, however, whether the birds persist at lower elevations because malarial transmission rates are low or whether individuals are developing immunogenetic resistance to the parasite through pathogen-driven selection (van Riper et al., 1986; Jarvi et al., 2000).

Recent studies of the pathogenicity of

sporozoite-induced experimental malarial infections in Iiwi (*Vestiaria coccinea*) and Apapane have shown that a single infective mosquito bite can cause significant morbidity and mortality in these species from fulminating erythrocytic infections (Atkinson et al., 1995; Yorinks and Atkinson, 2000). Mortality was lower in experimentally-infected Apapane, suggesting that the wider elevational range of this species may be related to individual differences in ability to recover from acute infections. In this study, we evaluate morbidity and mortality in experimentally-infected Hawaii Amakihi, using the same procedures and parasite isolates described in previous studies (Atkinson et al., 1995).

MATERIALS AND METHODS

Adult Hawaii Amakihi were captured with mist nets in September, 1993 in the Mauna Kea State Forest Reserve approximately 2 km west of Pu'u Koohi, Hawaii (19°45′N, 155°31′W, elevation 2,000 m). Birds were captured at three sites, spaced approximately 1 km apart. This area is dominated by dry, subalpine scrub land with scattered mamane (*Sophora chrysophylla*) and naio (*Myoporum sandwicense*) trees. Surveys of resident amakihi for malaria at this elevation on Mauna Kea have been consistently negative (C. T. Atkinson, unpubl. data). *Culex quinquefasciatus* has been reported at this elevation, but numbers are limited by cold temperatures, low humidity, and the absence of suitable larval habitats (Goff and van Riper, 1980).

Thirty two amakihi were captured, banded, weighed, and aged by plumage characteristics and either held overnight in temporary holding cages with food and water or transported within several hours of capture to a mosquito-proof aviary at Hawaii Volcanoes National Park. The birds were housed within the aviary in individual cages measuring 60 × 30 × 30 cm and maintained *ad libitum* on a diet of Nectar Plus (Necton Corporation, Clearwater, Florida) and slices of fresh oranges. Natural light cycles were maintained through a translucent corrugated fiberglass roof.

Approximately 100 μl of blood were collected from each bird within several days of capture via jugular venipuncture with a heparinized 28 gauge insulin syringe. Thin blood smears were fixed with methanol, stained for 1 hr with 6% phosphate buffered Giemsa, pH 7.0, and scanned at 400× for 10 min to confirm that birds did not have patent malarial infections. Remaining heparinized blood was spun in a microhematocrit centrifuge for collection of plasma. Plasma from each bird was tested by an immunoblot technique described by Atkinson et al. (1995) for antibodies to a crude erythrocyte extract of *P. relictum* to confirm that birds did not have chronic malarial infections.

Amakihi were acclimated to captivity for 4 wk and then assigned randomly to treatment ($n = 20$) and control ($n = 12$) groups. Each bird in the treatment group was exposed individually to the bite of a single *P. relictum*-infected mosquito. Colonized *Culex quinquefasciatus* and procedures described by Atkinson et al. (1995) were used to infect the amakihi. Birds in the control group were exposed to the bite of a single uninfected mosquito. Mosquitoes were infected from a Common Canary (*Serinus canarius*) that had been inoculated with a thawed, deglycerolized aliquot of the same Apapane isolate of *P. relictum* (KV-115) used in prior experimental studies (Atkinson et al., 1995; Yorinks and Atkinson, 2000). The aliquot that was used was passaged once in a canary after initial isolation in 1992, stored frozen, thawed, and passaged twice more in canaries before they were exposed to mosquitoes.

Amakihi were weighed and bled via the brachial vein for preparation of thin blood smears every 4 days beginning on the day they were exposed to mosquitoes (day 0) and continuing for 60 days. Thin blood smears were fixed and stained as described earlier and parasitemia was quantified by counting the number of infected erythrocytes per 1,000 erythrocytes as described by Godfrey et al. (1987). Nectar consumption was recorded daily between 0700 and 0900 for each bird.

Birds that died during the course of the experiment were refrigerated and necropsied within 24 hr of death. Representative pieces of all major organs were fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

Statistical analyses were performed as described by Atkinson et al. (1995). In brief, data on weight and food consumption were analyzed with repeated measures ANOVAs (Kirk, 1982) where responses for each bird were measured daily (food consumption) or at 4 day intervals (weight). In each analysis, treatment group, sex, and treatment-sex interactions were analyzed as among-block (bird) effects. Changes in responses over time and the interaction of time with treatment and sex were analyzed as within-block effects. Significant among-block effects were further analyzed using *t*-tests for multiple comparisons. Within-block effects were analyzed using linear contrasts. All ana-

lyses were conducted using general linear model procedures with Type III sums of squares (SAS, 1990). Statistical comparisons of data on food consumption and weight were limited to the first 13 days of the experiment for food consumption and 16 days for weight because mortality was high in the treatment group exposed to malarial infection. Three birds died from complications other than malaria during the experiment and were excluded from the analysis of weight and food consumption. Results of statistical tests were considered significant when $P \leq 0.05$.

Survival distributions between the two treatment groups were analyzed using a Kaplan-Meier estimator and the log-rank test (Cox and Oakes, 1984). The log logistic distribution (Cox and Oakes, 1984) was used to analyze the effects of initial weight and sex on survival because mortality was delayed until after birds developed patent infections and then declined as birds recovered from infection. The three birds that died from complications other than malaria were censored from the survival analyses on the day that mortality occurred (Samuel and Fuller, 1994).

RESULTS

Parasitemia and mortality

None of the amakihi used in the experiment had evidence of prior exposure to malaria by either microscopy or serology. All birds exposed to single infective mosquito bites developed patent infections by 8 days post-infection (PI). Salivary glands from the infected mosquitoes had sporozoite loads that were comparable in intensity to those observed in naturally-infected wild *Culex*, indicating that experimental birds received infective doses that were comparable to minimal levels of exposure in the wild.

Parasitemia in seven of the 20 infected amakihi peaked at 12 days PI at approximately 15% and slowly declined. Parasitemia in the remaining 13 birds continued to increase and reached intensities as high as 50% before these individuals died (Fig. 1). Overall mortality among infected amakihi was 65% (13/20), while none of the control birds died during the course of the experiment. Nine of the 13 fatalities died from acute malaria. A single bird survived the initial crisis at 12 days PI, but succumbed at 48 days PI when the infection recrudesced. Deaths of the remaining three birds were complicated by other factors; one bird died on day 16 PI from acute malaria complicated by a concurrent aspergillosis infection; one bird died on day 32 PI from apparent stress following routine handling and bleeding; one bird was euthanized on day 47 PI because of complications associated with an eye infection.

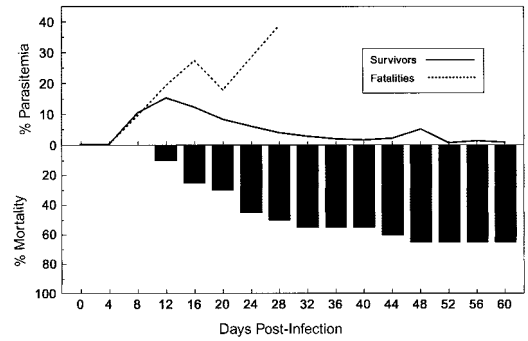

FIGURE 1. Parasitemia (top) and accumulated mortality (bottom) in experimentally-infected Hawaii Amakihi. Birds that were able to control parasitemia in acute infections and survive the initial crisis were classified as survivors.

Based on the Kaplan-Meier method, survival distributions differed significantly between the treatment and control groups (log-rank test, $\chi^2 = 9.90$, 1 df, $P = 0.002$). Mean survival time ($\pm$SD) for the nine fatalities that died from acute malaria was 21.3 ± 5.96 days (Range = 14–31 days; Median = 20 days) (Fig. 2). Parasitemia at death ranged from a low of 19% to a high of 50%.

The effects of gender and initial weight on survival were analyzed with a log-logistic model with weight as a continuous variable. Neither gender ($\chi^2 = 1.59$, 1 df, $P = 0.112$) or pre-infection weight had significant effects on survival ($\chi^2 = 1.26$, 1 df, $P = 0.209$).

Gross and microscopic pathology

The 10 amakihi that died from uncomplicated malaria had a variety of common gross lesions, including enlargement and

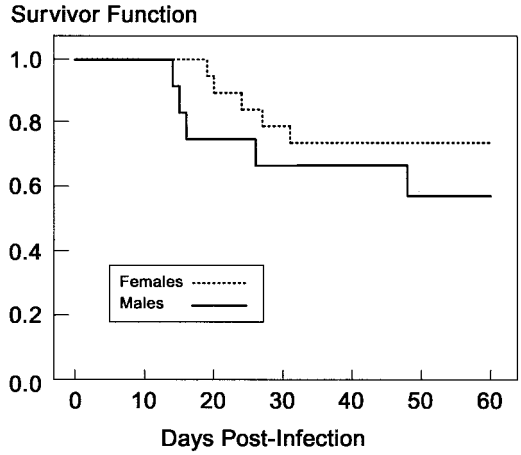

FIGURE 2. Kaplan-Meier survival curves for male (n = 8) and female (n = 12) Hawaii Amakihi. The three amakihi that died from complications other than malaria (two females and one male) were censored in the survival analysis and are not included in this graph.

blackish discoloration of the liver and spleen and presence of pale and watery heart blood. Other internal organs typically appeared normal. Birds that died prior to 21 days PI had some trace deposits of subcutaneous fat and were in relatively good flesh with only minor atrophy of the pectoral muscles. Four of five amakihi that died after 21 days PI were emaciated and had prominent keels.

Common microscopic changes among the ten uncomplicated malaria fatalities were mild to moderate in intensity and included diffuse pigment deposition in Kupffer cells of the liver and macrophages of the spleen. Diffuse areas of extramedullary erythropoiesis were present in sinusoids of the liver and interstitium of the kidneys. Multifocal, mild extramedullary granulopoiesis was found occasionally in hepatic parenchyma, in red pulp and subcapsular areas of the spleen, and in perivascular areas of the kidneys. Minimal to moderate interstitial pneumonia was evident in most birds with increases in numbers of granulocytes in the pulmonary interstitium.

In amakihi with the highest parasitemias at death, large numbers of immature erythrocytes were present in the circulation. Bone marrow in most birds exhibited increased cellularity with erythrocyte depletion. The granulocyte to erythrocyte ratio ranged from 1:1 to as high as 4:1.

Food consumption

Food consumption differed between males and females ($F = 12.5$; 1, 24 df; $P = 0.002$) with females consuming less than males. Differences between the two treatment groups were significant during the first 13 days of the experiment ($F = 5.3$; 1, 24 df; $P = 0.03$) with no significant interaction between treatment group and sex ($F = 3.5$; 1, 24 df; $P = 0.074$). Food consumption changed significantly over time ($F = 9.1$; 13, 312 df; $P < 0.0001$), between groups over time ($F = 3.4$; 13, 312 df; $P < 0.0001$), but did not vary between sexes over time ($F = 1.2$; 13, 312 df; $P = 0.315$) or according to sex-group combinations ($F = 0.9$; 13, 312 df; $P = 0.574$). Infected amakihi showed significant declines in food consumption during the first 13 days of the experiment (linear contrasts, $P < 0.0001$). Food consumption by uninfected control birds did not change significantly ($P = 0.225$) during this period.

Infected amakihi were reclassified according to survival status, i.e. ability to recover from acute infection, as either survivors or fatalities and food consumption data were reanalyzed. Slopes of food consumption over time during the first 13 days of the experiment were marginally different when comparisons were made between control and surviving birds ($F = 3.7$; 1, 165 df; $P = 0.057$) and significantly different when comparisons were made between control birds and fatalities ($F = 14.5$; 1, 165 df; $P < 0.0001$). Slopes of food consumption did not differ significantly between surviving birds and fatalities during the same period ($F = 3.3$; 1, 123 df; $P = 0.072$) (Fig. 3). When the analysis was extended to include the entire 60 day period of the experiment, food consumption for control birds increased slightly during the course of the experiment (linear con-

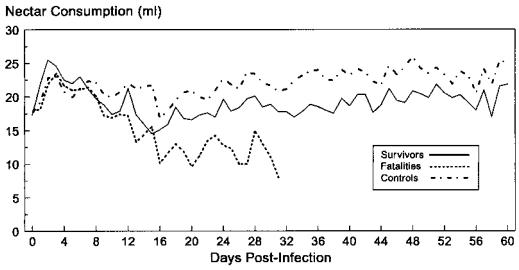

FIGURE 3. Mean daily nectar consumption (m1) for survivors (n = 8), fatalities (n = 9) and uninfected control amakihi (n = 12). Three infected amakihi were excluded from the analysis because they died from complications other than malaria.

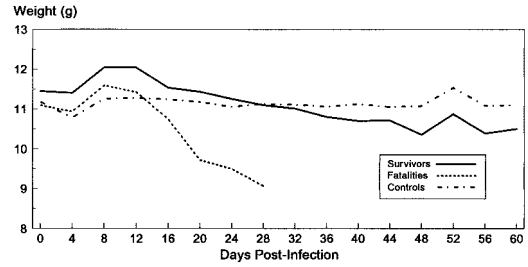

FIGURE 4. Mean weights (g) for survivors (n = 8), fatalities (n = 9) and uninfected control amakihi (n = 12). Three infected amakihi were excluded from the analysis because they died from complications other than malaria.

trasts, $P < 0.0001$). Food consumption for surviving amakihi exhibited a significant quadratic trend during the experiment, decreasing until 16 Days PI and then increasing as birds recovered from infection (linear contrasts, $P < 0.0001$) (Fig. 3).

Weight

Mean (±SD) weights for males ($\bar{x}$ = 11.99 ± 0.921 g, n = 12) were significantly heavier than females ($\bar{x}$ = 10.64 ± 0.694 g, n = 18) ($F = 14.3$; 1, 23 df; $P = 0.001$), but treatment ($F = 0.2$; 1, 23 df; $P = 0.636$) and treatment-sex interactions ($F = 0.001$; 1, 23 df; $P = 0.972$) were not significant. Within-block effects were significant for time ($F = 16.8$; 4, 92 df; $P < 0.0001$) and time-treatment interactions ($F = 7.1$; 4, 92 df; $P < 0.0001$), indicating that there were differences in how weights in the treatment and control groups changed over time. No other differences related to interactions among time, sex, or treatment group were significant ($P > 0.251$).

Amakihi were reclassified according to survival status and mean weights for control birds, survivors, and fatalities were compared within each group over the entire 60 day period of the experiment (linear contrasts). Mean weights for control birds did not change over time ($P = 0.984$), while weights for both fatalities and survivors declined ($P < 0.0001$). Slopes of regression lines for all three groups were significantly different ($P \leq 0.0001$) (Fig. 4). Weight loss was correlated with survival status, with an average decline of 13% from pre-infection body weights for fatalities, an average decline of 5% for survivors and an average increase in weight of <1% for control birds (Fig. 4).

DISCUSSION

Results from this study are similar to those from other experimental studies of malaria in native Hawaiian forest birds (van Riper et al., 1986; Atkinson et al. 1995; Yorinks and Atkinson, 2000). Infected amakihi experienced declines in food consumption and body weight during the course of the experiment. We suspect that the primary cause of death among birds with uncomplicated malarial infections was anemia associated with the erythrocytic infections, but were not able to obtain enough blood from these acutely ill individuals to measure hematocrit. Other lesions that were common in all of these birds support this diagnosis, including presence of pale, watery heart blood, high parasitemias with abundant immature erythrocytes, and diffuse areas of extramedullary erythropoesis in the liver and kidney.

Atkinson et al. (1995) observed a significant relationship between survivorship and both gender and initial weight in Iiwi with experimental malarial infections and suggested that the immunosuppressive ef-

fects of testosterone might be responsible for lower survivorship of male birds. Iiwi with heavier initial weights survived longer than lighter birds, suggesting that overall body condition is a good predictor of ability to survive acute infections. We failed to find statistically significant relationships between these variables and survivorship in this study, even though the general trends were similar. Female amakihi survived slightly longer than males and mean weights of surviving amakihi were slightly larger than either controls or fatalities until after the crisis (Fig. 4), but small sample sizes may have contributed to the failure to find statistically significant differences.

Declines in food consumption occurred during the first 13 days of the experiment as peripheral parasitemias increased in infected birds, but these declines were not reflected as a loss of body weight until after the crisis (Figs. 3, 4). Amakihi that died from acute malaria began to experience declines in food consumption at approximately 8 days PI that continued until death. By contrast, birds that recovered from the crisis when parasitemia peaked began to increase food consumption, but continued to lose weight throughout the 60 day course of the experiment.

It is significant that surviving amakihi were acutely ill and experienced losses of up 5% of their body weights during this experiment. Significant declines in body weight, fat levels, and locomotory behaviors in Apapane that survived experimental malarial infections have been documented, suggesting that these birds would be particularly susceptible to non-native predators (e.g., black rats, *Rattus rattus*; house cats; and mongooses, *Herpestes aureopunctatus*) during acute phases of the infection (Yorinks and Atkinson, 2000). Both experiments support the idea that native honeycreepers face intense selective pressure in areas with active malaria transmission. Failure to recover lost weight may place chronically infected individuals at a competitive disadvantage in mosquito-free, high elevation habitats where thermal stresses are greater (Hayworth et al., 1987). This disadvantage would be balanced at lower elevations, however, by acquired concomitant immunity to the infection, possibly giving chronically-infected individuals a competitive edge in habitats where disease transmission is more stable.

Jarvi et al. (2000) hypothesized that the ability to survive malarial infection in Hawaiian honeycreepers is correlated with diversity of major histocompatibility complex (*Mhc*) genes and that it may be possible to detect elevation-dependent differences in specific *Mhc* alleles or allele frequencies if these genes play an important role in mediating humoral and cell mediated responses to the infection. These authors found much lower *Mhc* diversity in Iiwi than Hawaii Amakihi using probes to the antigen binding region of Class II (beta chain) *Mhc* genes in southern blots and suggested that the prolonged, high parasitemias observed in birds that succumbed to malarial infection could possibly be explained by failure of the immune system to recognize and process specific malarial-encoded peptides (Jarvi et al., 2000). Interestingly, gross and microscopic lesions in Iiwi infected with the same isolate of *P. relictum* under similar experimental conditions were much more severe than those we observed in amakihi that succumbed to infection. These observations provide some additional circumstantial evidence that immunogenetic factors may be important in determining outcome from infection, but many loci and genetic systems in addition to the *Mhc* could be involved.

We were able to demonstrate that some Hawaii Amakihi collected from high elevation habitats have the physiological capacity to survive malarial infection. While mortality was high (65%), it was lower than what was observed in experimentally-infected Iiwi (90%) (Atkinson et al., 1995) and more similar to mortality in Apapane (63%) collected from similar high elevation locations (Yorinks and Atkinson, 2000). Thus, the wider altitudinal range and geographic distribution of both Ha-

waii Amakihi and Apapane may be related to ability to survive malarial infections.

Van Riper et al. (1986) compared susceptibility of Hawaii Amakihi collected in high elevation mesic habitats on Mauna Loa Volcano with amakihi from the same xeric habitat used in this study to look for differences in resistance to infection. Two of five Mauna Loa amakihi were completely refractory to an intramuscular injection of infected blood, two developed patent infections and recovered, and one bird died from acute malaria. By contrast all Mauna Kea amakihi developed patent infections and three of five (60%) succumbed. The authors speculated that the greater continuity of wet forest habitats on Mauna Loa has facilitated flow of genes responsible for disease resistance between low and high elevation populations, making amakihi populations in that region of the island more resistant to the disease. We did not observe any evidence of refractoriness in the comparatively large sample of high elevation Mauna Kea amakihi that we exposed to malaria by mosquito bite. We have encountered apparent refractoriness to malaria in other honeycreepers that we have infected in the laboratory, but only when individuals had chronic, low level infections that were not readily detectable by blood smears (C. T. Atkinson, unpubl. data). Since van Riper et al. (1986) did not have the means to confirm that their birds were serologically negative for avian malaria, it is possible that the two refractory amakihi described in that study actually had acquired concomitant immunity to reinfection rather than genetic refractoriness to infection. Another possible explanation is that the intramuscular route of inoculation used in the study is not as efficient as mosquito bite in transmitting the infection (Vaughan et al., 1999). This question can only be resolved through controlled experimental infections using uninfected birds from areas where malaria transmission is now endemic.

The fact that some high elevation native honeycreepers have the ability to survive malarial infections bodes well for the long-term survival of these species, but this optimism must be tempered with the observation that others, like the Iiwi, appear to have virtually no resistance to the disease (Atkinson et al., 1995). Failure to incorporate this knowledge into captive propagation and translocation protocols can lead to catastrophic loss of the entire cohort of released birds if it coincides with seasons and locations where malaria transmission is endemic (Kuehler et al., 1996). If vector control techniques prove to be impractical in remote forest habitats, one of the great challenges facing natural resource managers in Hawaii will be development of effective techniques for identifying more disease-resistant individuals for restoration of self-sustaining, low-elevation populations.

ACKNOWLEDGMENTS

We thank D. Xiang and M. Samuel for their advice and assistance with statistical analysis of the data, D.J. Forrester and T. Pratt for helpful comments on earlier versions of the manuscript and J. Coatesworth, J. Mills, and J. Wilcox for help with capturing and caring for experimental birds. Funding for this project was provided by the United States Geological Survey—Biological Resources Division.

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Received for publication 5 April 1999.