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Costs of Resistance to Natural Enemies in Field Populations of the Annual Plant *Arabidopsis thaliana*

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ABSTRACT: The annual plant *Arabidopsis thaliana* is widely used as a model system in molecular genetics, but little is known about populations in the field. In this experimental field study of natural populations of *Arabidopsis,* I tested the assumption that plant resistance has fitness costs. Models of the evolution of resistance assume a cost, which is envisioned as a reduction in fitness in the absence of natural enemies, such as insect herbivores and pathogens. The presumed basis of this cost is the diversion of limiting resources away from present and future growth and reproduction. Recent failures to detect allocation costs of resistance to herbivores have raised questions about whether costs exist and, thus, about the appropriateness of theories that postulate such costs. I found genetic variation for two traits commonly thought to function as resistance characters: trichome density and total glucosinolate concentration. Under field conditions, these characters both reduced damage by the natural assemblage of herbivores and exhibited significant fitness costs.

Keywords: Arabidopsis thaliana, cost, defense, herbivores, pathogens, resistance.

Costs play a central role in ecological and evolutionary theory, most notably in the area of life-history evolution, because the fitness costs associated with a character determine its equilibrium value. In particular, most models of the evolution of resistance to herbivores make the assumption that resistance has a fitness cost (Gulmon and Mooney 1986; Fagerström et al. 1987; Simms and Rausher 1987). The presumed basis of this cost is the diversion of limiting resources away from present and future growth and reproduction (Bazzaz et al. 1987).

A primary motivation for incorporating costs into models of the evolution of resistance is that costs can account for the common observation that, although plants exhibit considerable genetic variation for resistance (Dirzo and Harper 1982; Berenbaum et al. 1986), they are not maximally resistant to attack by herbivores and pathogens. Since such natural enemies can reduce the fitness of the plants on which they feed (Morrow and LaMarche 1978; Marquis 1984; Burdon 1987) and resistance characters, by definition, protect plants against damage by herbivores (Fraenkel 1959; Berenbaum 1978; Blau et al. 1978), most plants should experience directional selection for increased resistance. In the absence of costs, such selection could eventually make all plants highly resistant and eliminate variation for resistance. Costs can lead to an evolutionary equilibrium at intermediate levels of resistance by counteracting the benefits of reducing herbivore damage (Simms and Rausher 1987; Mauricio and Rausher 1997).

Recent failures to detect fitness costs of resistance to herbivores and pathogens (Simms and Rausher 1987; Brown 1988; Ågren and Schemske 1993) have raised questions about whether such costs exist in natural plant populations (Simms 1992; Simms and Triplett 1994) and, thus, about the appropriateness of theories that postulate such costs. Here, I demonstrate the existence of fitness costs of resistance in natural populations of the annual plant *Arabidopsis thaliana.*

Methods

Natural History

Arabidopsis thaliana (L.) Heynh. (Brassicaceae) is a predominantly self-fertilizing winter annual plant commonly found in disturbed habitats (Ratcliffe 1961; Jones 1971; Snape and Lawrence 1971; Baskin and Baskin 1972). The plant possesses two characters commonly thought to provide resistance to herbivores: trichomes cover the leaves and stems, and the leaves and seeds contain glucosinolates, a class of natural products characteristic of the family Brassicaceae (Vaughan et al. 1976; Hogge et al. 1988).

The most common herbivores I have collected on *A. thaliana* in North Carolina are the flea beetles, *Psylliodes*

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convexior LeConte and *Phyllotreta zimmermani* Crotch (Chrysomelidae), although I have also observed aphids, leaf miners, and the larvae of a geometrid moth feeding on the plants in the field. Several fungal and bacterial pathogens are reported to occur on natural populations of *A. thaliana* (Morgan 1971; Koch and Slusarenko 1990; Dangl et al. 1992; Tsuji and Somerville 1992).

I collected seeds from 144 individual plants growing in Durham County, North Carolina. Plants were collected from four sites separated by at least 1 km. The population structure for *A. thaliana* in this area is not known.

Since *A. thaliana* is predominantly selfing, seeds from each plant represent an inbred line, or family. All experimental plants within a family were taken from a single maternal parent. Differences among families represent both genetic differences and effects of common (maternal) environment. Because each family represents an inbred line, any among-family genetic variance or covariance detected in this experiment represents total, rather than additive, genetic variance or covariance.

Estimates of total genetic variance and covariance are appropriate for understanding evolution in this species, however, for two reasons. In a population of selfing organisms, natural selection acts on the total genetic variation and not exclusively on the additive genetic variation, as is the case for outcrossing species (Roughgarden 1979). Also, the crosses that would be used to partition total genetic variation into additive and nonadditive components would generate offspring with artificially high heterozygosity and, thus, would be of questionable relevance to evolution in natural populations of *A. thaliana.* In order to minimize environmentally derived differences among offspring of different plants (e.g., maternal effects), I propagated families used in this experiment in a growth chamber for three generations.

Experimental Design

On December 7, 1993, I transplanted 1,728 young plants (at the two-to-four true leaves stage) to a field in the Catsburg region of Durham County, North Carolina, where *A. thaliana* grows naturally and at a time when natural populations of *A. thaliana* were at a similar phenological stage. The field site was located close to the original collection sites. Natural vegetation within the experimental area was not disturbed.

Half of the plants were exposed to the natural assemblage of herbivores and pathogens and the other half were sprayed with pesticides to eliminate natural enemies. Plants were sprayed with 40 μL of the insecticide esfenvalerate (ASANA XL, Dupont, Wilmington, Del.) per liter of water, 1 g of the fungicides metalaxyl and chlorothalonil (Ridomil/Bravo 81W, Novartis, Greensborg, N.C.) per liter of water, and 1 g of the fungicide benomyl (Benlate, Dupont, Wilmington, Del.) per liter of water, at approximately 2-wk intervals until the completion of the experiment. Pesticides were applied on three consecutive days so that only one pesticide was sprayed on any day. Because of the possibility of contamination of controls by pesticides, treatments were spatially separated using a split plot design, with three spatial blocks each consisting of two control and two sprayed subplots. One individual from each of the 144 families was randomly assigned to each treatment subplot.

Between March 14 and 23, 1994, before plants began to flower, I collected four entire leaves from each plant and measured the leaf area, trichome density, and concentration of total glucosinolates for each sample. All sampled leaves were fully expanded and approximately the same age. Such leaf sampling might reduce plant fitness, but the proportional reduction in fitness should be similar for all plants. Leaf samples were immediately and stored on ice. Within 12 h of collection, the areas of the leaves were measured using a video-imaging system, and glucosinolates were extracted from two leaves by boiling in water for 7 min, followed by grinding. The resulting solution was stored at −80°C until analysis. For consistency, trichomes were counted within a 2.4-mm^2 area of the distal central portion of the adaxial (upper) surface of two leaves. Trichomes are uniformly distributed on the upper leaf surface of *A. thaliana.*

Total glucosinolates—expressed as the amount of glucose (milligrams per liter) released by enzymatic hydrolysis of glucosinolates (1 glucose = 1 glucosinolate) per milligram leaf (wet weight)—were assayed using the micro-column method (Heaney and Fenwick 1981). Glucose absorbance was measured using a spectrophotometer at 490 nm. Concentrations were estimated using a dextrose standard curve. The wet weight of each leaf was estimated using a regression of leaf area and wet leaf weight previously determined from a sample of field-collected leaves (leaf weight = 0.153 × leaf area − 0.414; $r = 0.97$; $N = 100$ leaves).

Before rosettes senesced, plant size was estimated by measuring the diameter of the leaf rosette. Rosette diameter is a good predictor of rosette area, a measure of plant size (rosette diameter = 0.33 × rosette area + 1.7; $r = 0.93$; $N = 76$ plants). From April 9 to 12, 1994, plants in the field were examined for signs of damage by herbivores. Herbivore damage was quantified as the total number of leaf holes made by insects. Damage holes were rather uniform in size, with an average area of 1.26 mm^2 and a standard deviation of 1.25. Average area of a single leaf, by contrast, was 40.5 mm^2 with a standard deviation of 32.3 (Mauricio et al. 1997).

By May 10, 1994, all plants had stopped flowering and all individuals were harvested. For each plant, I counted

the total number of fruits as a measure of fitness. Plants that died before fruiting were included in the analysis as having zero fitness. Since *A. thaliana* is a selfing annual, total fruit number represents both female and male reproductive effort. Total number of fruits is an excellent predictor of total seed number among field-collected individuals of *A. thaliana* (seed number = 40.5 × fruit number − 72.0; $r = 0.98$; $N = 50$ plants).

Statistical Analysis

All data were analyzed using procedures in the SAS statistical package (version 6.09). ANOVA and regressions were calculated using the General Linear Model procedure. Broad-sense genetic correlations among characters were estimated from the among-family components of variance and covariance using the NESTED procedure (Via 1984). The NESTED procedure performs a random effects analysis of variance (SAS Institute 1990). The variance and covariance components were partitioned into among-family (family) and within-family (error) terms. The estimate of the genetic correlation as a result of family was calculated as the ratio of the appropriate family covariance component to the square root of the product of the family variance components for the two different dependent variables (SAS Institute 1990).

Since the sampling variance of genetic correlations is unknown, both Becker (1992) and Falconer and Mackay (1996) urge caution in estimating their standard errors. Many authors have recommended that the standard errors of such parameters as heritabilities and genetic correlations be estimated using the jackknife resampling procedure (Cohen 1969; Arvesen and Schmitz 1970; Efron 1982; Roff and Preziosi 1994).

In the jackknife procedure, the genetic correlation is iteratively calculated as described above. At each iteration, a family is omitted from the analysis of variance and a new correlation is calculated. Pseudovalues, θ_i, are calculated as

$$\theta_i = k\rho - (k - 1)\rho_{-i},$$

where k is the number of families, ρ is the genetic correlation calculated from all 144 families, and ρ_{-i} is the genetic correlation calculated with the ith family deleted. The average of the pseudovalues, θ, gives a nearly unbiased estimator of the genetic correlation with a standard error of

$$SE_\theta = \sqrt{\frac{\Sigma (\theta - \theta_i)^2}{k(k - 1)}}.$$

The statistical significance of the genetic correlation was assessed by calculating the 95% confidence interval and observing if the interval overlaps zero.

Effect of Pesticides

In a separate experiment, I showed that this regime of pesticide application did not affect the performance of *A. thaliana.* A total of 288 plants (two treatments applied to eight plants from each of 36 randomly selected families) were planted in trays and placed in a growth chamber (12-h day length at 20°C). Half of the plants were sprayed with esfenvalerate, metalaxyl/chlorothalonil, and benomyl, and the other half were sprayed with deionized water. Pesticides were applied at 2-wk intervals, on three consecutive days, so that only one pesticide was sprayed on any day. The amount of pesticide applied to each plant matched the amount applied in the field experiment. After senescence at 14 wk, I counted the total number of fruits on each plant. There was no significant effect of the treatment on number of fruits produced (ANOVA; $F = 2.84$; df = 1, 96; $P = .09$; the error degrees of freedom were reduced by additional effects in the ANOVA). Sprayed plants produced a mean of 78 fruits (SE = 6.2), and unsprayed control plants produced an average of 93 fruits (SE = 6.0).

Induced Resistance

In an additional experiment, I found that trichomes and glucosinolates were not induced by damage. This experiment was necessary because, if resistance is inducible, plants from which natural enemies were eliminated would remain uninduced and might not incur the full costs of resistance. A total of 140 plants (two treatments applied to 20 plants from each of seven randomly selected families) were propagated in a growth chamber (12-h day length at 20°C). Within each family, plants were randomly paired. Half of the plants were damaged by pricking all leaves in the rosette with a needle; such mechanical wounding has been shown to induce glucosinolates in the related species *Brassica rapa* and *Brassica juncea* (Bodnaryk 1992). The damage treatment was administered 1 wk and 5 wk after transplantation. Five days after the second damage treatment, I collected four undamaged leaves from all plants and measured the total glucosinolate concentration and trichome density. Damage did not cause a significant change in the levels of either glucosinolate concentration (paired t-test; $t = -0.45$; $P = .65$) or trichome density (paired t-test; $t = 0.81$; $P = .42$).

Results

Spraying with pesticides almost completely eliminated leaf damage by the assemblage of natural enemies present in the field (ANOVA; $F = 396.3$; df = 1, 854; $P = .0001$). Sprayed plants had a mean of 0.2 damage holes

(SE = 0.036; 1.6% of their leaves damaged) while control plants experienced an average of 3.3 holes (SE = 0.189; 21.6% of their leaves damaged). Thus, the spraying regime was very effective in reducing visible herbivore damage. I was unable to determine if the pesticides effectively reduced damage by cryptic herbivores (e.g., aphids) and pathogens.

In the field, natural enemies had a strong detrimental effect on plant fitness (Mauricio et al. 1997). Plants from which natural enemies had been removed produced significantly more fruits ($\bar{X}$ = 217 fruits; SE = 5.0) than plants that experienced natural levels of damage ($\bar{X}$ = 160 fruits; SE = 6.7; ANOVA; F = 56.3; df = 1, 854; P = .0001). This effect does not seem to be a result of the action of the pesticides since the growth chamber experiment on the effects of pesticide showed no statistically significant effect. The trend observed in that experiment indicated, if anything, a lower mean fruit number in sprayed plants than in the controls. Therefore, the effect on fruit number observed in the field experiment is in the opposite direction as would be predicted if the effect was a result of the pesticides.

Resistance characters are, by definition, traits that reduce the amount of damage an individual plant experiences. In this experiment, I was able to assess this prediction for herbivores leaving visible leaf damage. In the control plots, families with higher levels of both trichome density and total glucosinolate concentration experienced less leaf damage than families with lower levels of these characters. Trichome density is negatively correlated with leaf damage (broad-sense genetic correlation = −0.1635; SE = 0.0789; $P < .05$), as is glucosinolate concentration (broad-sense genetic correlation = −0.1981; SE = 0.0780; $P < .05$).

There is a significant positive correlation between total glucosinolate concentration and trichome density (broad-sense genetic correlation = 0.3814; SE = 0.1157; $P < .001$; phenotypic correlation = 0.2145; $P < .0001$; Mauricio and Rausher 1997). Therefore, I performed a multiple regression analysis on phenotypic values where I included both trichome density and glucosinolate concentration in the model. This analysis also shows a significant negative phenotypic relationship between trichome density and leaf damage (F = 21.8; df = 1, 837; $P < .0001$) in the field. Although the phenotypic relationship between leaf damage and glucosinolate concentration only approaches statistical significance (F = 3.3; df = 1, 837; P = .06), it suggests that glucosinolates are also acting as resistance characters.

Because assessment of costs of resistance requires the presence of genetic variation for the characters, I determined that genetic variation for resistance existed in these plants. An ANOVA reveals family-level variation for both resistance characters (table 1). Therefore, both trichome density and total glucosinolate concentration exhibit significant genetic variation.

Both trichomes and glucosinolates exhibit significant costs. To assess costs, I used the standard method of determining whether there is a significant negative genetic correlation between the resistance character and fitness in the absence of natural enemies (Reznick 1985; Berenbaum et al. 1986; Simms and Rausher 1987). The logic underlying this method is that when natural enemies are present, the relationship between resistance and fitness reflects both the cost and the benefit of resistance (the reduction in damage). By eliminating natural enemies, the benefit of resistance is removed (Mauricio and Rausher 1997), and the relationship between resistance and fitness reflects only the cost of resistance. In *Arabidopsis thaliana,* in the absence of natural enemies, I found a significant negative genetic correlation between trichome density and fruit number (broad-sense genetic correlation = −0.4558; SE = 0.0704; $P < .0001$) and between glucosinolate concentration and fruit number (broad-sense genetic correlation = −0.2667; SE = 0.0725; $P < .001$).

Again, since there is a significant positive correlation between total glucosinolate concentration and trichome density, I performed a multiple regression analysis on phenotypic values in which I included both trichome density and glucosinolate concentration in the model (see Mauricio and Rausher 1997 for a comparable analysis on genotypic values). This analysis also shows a significant negative phenotypic relationship between trichome density and fruit number (F = 90.7; df = 1, 828; P = .0001) and between fruit number and glucosinolate concentration (F = 3.6; df = 1, 828; P = .05).

Discussion

The results of the experiments reported here provide some of the first definitive evidence that characters that confer resistance to natural enemies exhibit fitness costs under natural conditions. In particular, such costs were detected for both a morphological character and a suite of plant natural products in field populations of *Arabidopsis thaliana.* These costs suggest that the evolution of both characters may be constrained by genetic trade-offs.

Negative correlations such as these may be a result of either pleiotropy or linkage disequilibrium (Falconer and Mackay 1996). Usually only those resulting from pleiotropy are considered true costs because, in outbreeding species, genetic correlations resulting from linkage disequilibrium are expected to decay over time with recombination (Lewontin 1974; Hartl and Clark 1989). Thus, correlations resulting from linkage disequilibrium are not assumed to represent a permanent constraint on the evolution of resistance. However, in a highly selfing species such as *A. thaliana,* effective recombination is probably

Table 1: ANOVA for trichome density and total glucosinolate concentration of *Arabidopsis thaliana* grown in the field

	Trichome density				*Glucosinolate concentration*			
Source of variation	*df*	*Type III sums of squares*	F	P	*df*	*Type III sums of squares*	F	P
Family (site)*	140	30,822.12	1.36	.0061	140	1,094.02	1.27	.0251
Site	3	1,324.87	2.73	.0428	3	18.93	1.03	.3791
Treatment	1	2,439.33	15.49	.0001†	1	97.45	15.61	.0001†
Block	2	17,249.35	54.78	.0001†	2	20.11	1.61	.2015†
Treatment × block	2	1,886.32	5.83	.0030	2	7.39	.60	.5478
Treatment × site	3	203.91	.42	.7384	3	58.96	3.20	.0227
Block × site	6	1,231.67	1.27	.2687	6	44.18	1.20	.3037
Treatment × block × site	6	818.84	.84	.5359	6	119.66	3.25	.0036
Treatment × family (site)	140	15,456.00	.68	.9974	140	1,098.18	1.28	.0234
Block × family (site)	278	43,766.57	.97	.6001	284	1,735.00	1.02	.4230
Treatment × block × family (site)	275	33,201.90	.75	.9980	275	2,048.26	1.21	.0217
Error	836	135,148.38	...	...	826	5,065.94	...	...

* The family term is nested within collection site.

† Split-plot design requires the test of this hypothesis using the mean square for the block × family (site) term as the error (Snedecor and Cochran 1967; Freund et al. 1986).

uncommon. Because genes in linkage disequilibrium may be held together stably, resistance and its apparent costs would tend to be inherited together as well. Thus, costs will constrain the evolution of resistance in the same way as they would if the costs were actually a result of pleiotropy.

Given the breeding system of *A. thaliana,* it is possible that the genetic variation and covariation I observed was a result of population differentiation rather than variation maintained within populations. Unfortunately, little is known about population structure in natural populations of *A. thaliana.* However, I attempted to address this issue by using the collection site as a surrogate for population in the ANOVA. A significant amount of variation in trichome density was explained by the collection site, although there was no evidence for among-site differences in glucosinolate concentration. Accounting for variation as a result of population, the ANOVA revealed significant among-family variation in both trichome density and glucosinolate concentration.

Many researchers have attempted to detect costs of resistance to herbivores in natural plant populations but they have not observed significant costs (Simms and Rausher 1987, 1989; Simms 1992; Ågren and Schemske 1993; Simms and Triplett 1994). Early attempts, based on nongenetic approaches, initially suggested that costs might be common. However, most of these approaches have not determined if there is a negative genetic correlation between resistance and fitness under natural field conditions. For example, although some investigators have calculated the cost of resistance in the currency of adenosine triphosphate (ATP), nicotinamide adenine dinucleotide phosphate (NADPH), or carbon (Penning de Vries et al. 1974; Chew and Rodman 1979), these estimates are not meaningful in an evolutionary context unless the costs can be expressed in the relevant units of plant fitness.

Others have inferred the existence of costs of resistance from the detection of significant negative phenotypic correlations between fitness and resistance (Hanover 1966; Cates 1975; Coley 1986; Baldwin et al. 1990). Such studies must be viewed with caution because environmental covariances may cause the phenotypic covariances to differ in both sign and magnitude from the underlying genotypic covariances (Reznick 1985; Rausher 1992). Finally, although genetic studies using plants grown under controlled environments, such as growth chambers or greenhouses, have suggested the existence of costs (Berenbaum et al. 1986; Han and Lincoln 1994), it is known that changes in correlations can occur when organisms are grown in novel environments (e.g., Service and Rose 1985).

By contrast, recent attempts to detect costs of resistance as negative genetic correlations with fitness have not found them (Simms 1992). However, this failure may be a result of reasons other than the actual absence of costs of resistance (Charlesworth 1990). For example, costs may not be revealed as a pairwise negative correlation between resistance and fitness because these two traits are mutually correlated with other characters

(Houle 1991). Costs may also go undetected because they may be manifested only under certain environmental conditions that differ from those used in experiments (Bergelson 1994).

Finally, recent genetic studies may not have had the statistical power to detect genetic correlations. Although a lack of power may in many cases simply be a result of limitations in sample size, the type of resistance character assayed may also make it difficult to detect trade-offs. For example, some investigators have based their measurement of resistance on the amount of damage a plant experiences (i.e., 1 − proportion of leaves damaged; Simms and Rausher 1987, 1989; Núñez-Farfán and Dirzo 1994). Although this definition integrates over all specific resistance characters and thus may be a preferable index of what we actually mean by resistance, there may be considerable error in its estimation. For example, plants that are, by chance, missed by herbivores will be scored as having high resistance whether they are actually palatable to herbivores or not. Likewise, plants that are repeatedly sampled by different individual herbivores but rejected by each because the plant is highly unpalatable might have considerable damage and be scored as having low resistance. Although adequate replication may allow a reasonable estimate of resistance, the error associated with such a measure may make it more difficult to detect significant genetic correlations.

A different type of explanation for failure of genetic studies to detect costs of resistance is provided by the suggestion of several authors that costs themselves may evolve: costs may be reduced by making resistance inducible by herbivore damage or by selection at epistatic modifier loci that ameliorate the costs (Simms 1992). For example, Lenski (1988) used the latter hypothesis to explain how populations of virus-resistant bacteria had significantly reduced the cost of resistance after 400 generations.

Although this model could explain the amelioration of some types of resistance costs, it should not apply to allocation costs, which arise because investment of resources in resistance necessarily reduces the resource available for growth or reproduction (Bazzaz et al. 1987). For example, mutations may arise that reduce the cost of resistance by modifying the efficiency of metabolic pathways; ultimately, however, the fact that the resource is limiting and can only be partitioned to one component should eventually prevent costs from being reduced further. Whether this assumption is biologically warranted deserves empirical attention.

In this study, I detected significant fitness costs for two distinct types of resistance characters: a morphological trait, trichome density, and a suite of natural products, the glucosinolates. Most plant species possess multiple resistance characters (Berenbaum 1985). Based on the assumption that there are costs of resistance, several authors have suggested that there should be a trade-off among resistance characters (Rehr et al. 1973; Steward and Keeler 1988; Björkman and Anderson 1990). There is some evidence for such a relationship. Rehr et al. (1973) found that *Acacia* species possessed either cyanogenic glycosides or symbiotic ant-based defenses but not both. Björkman and Anderson (1990) showed that a morph of a South American blackberry lacking glandular trichomes had significantly tougher leaves than a morph with trichomes.

In contrast, Steward and Keeler (1988) found no relationship between indole alkaloids and three physical resistance characters in 19 species of the genus *Ipomoea.* In natural populations of *A. thaliana,* I found a significant positive correlation between trichome density and total glucosinolate concentration.

A trade-off among resistance characters seems likely only when two resistance factors are redundant, that is, directed toward the same set of natural enemies. It is not known to what extent different types of resistance may have evolved in response to different sets of herbivores. Moreover, the interaction between insect herbivores and plant resistance characters are complex (Blau et al. 1978; Chew 1988). Specialist herbivores may overcome plant natural products (Ehrlich and Raven 1964) while still being deterred by morphological barriers. Thus, morphological resistance characters, such as trichomes, and plant natural products may be directed at protecting the plant from different sets of natural enemies.

My demonstration that fitness costs of resistance can be detected in natural plant populations under field conditions suggests that the incorporation of costs into models of the evolution of resistance is appropriate, at least under some circumstances. In addition, this work provides an ecological and evolutionary context for ongoing research in the molecular genetics of resistance in *A. thaliana,* an important model organism (Marks and Feldmann 1989; Hülskamp et al. 1994; Magrath et al. 1994).

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