BRIEF COMMUNICATIONS

Evolution, 57(6), 2003, pp. 1444–1447

SPECIFIC VERSUS NONSPECIFIC IMMUNE DEFENSE IN THE BUMBLEBEE, *BOMBUS TERRESTRIS* L.

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Abstract.—Hosts vary in both their strength of response to a general immunological insult and in their specific susceptibility to different parasite species or different strains of the same parasite. The variation in the general immune response is considered a result of the costs imposed by selection on defended individuals. The variation in the specific response may originate from variation in host and parasite genotypes and is a requirement for frequency-dependent selection. The relationship between these two fundamental aspects of defense has only rarely been studied. Using the bumblebee *Bombus terrestris* and its gut trypanosomal parasite *Crithidia bombi* we found that the host's specific response profile toward different strains correlates negatively with its level of response to a general insult. This is the opposite result one would expect if the level of general response were simply a measure of immunological quality (immunocompetence). Rather, it suggests that there is some form of a trade-off between these two fundamental aspects of the immune system. These results, therefore, shed an important light on the possible constraints that affect the evolution of the immune system and particularly the trade-off between different arms of the immune system.

Key words.—*Crithidia bombi*, encapsulation, insect immunity, trade-offs, trypanosomes.

Received December 3, 2002. Accepted January 9, 2003.

Hosts typically vary in the strength of their general, nonspecific (innate) immune response against parasites. Such variation has often been explained in terms of costs (Sheldon and Verhulst 1996) for the evolution (Kraaijeveld and Godfray 1997) or use (Moret and Schmid-Hempel 2000) of the immune system. Hosts also vary in the response of their specific immune responses when they are differentially susceptible to different species of parasites, or, as investigated in this article, to different strains of the same parasite (Schmid-Hempel et al. 1999; Carius et al. 2001). In current theories of host-parasite coevolution, specificity based on variation among strains within parasite species holds an important position, since it is a precondition to frequency-dependent selection on host populations. The resulting rapid coevolution in turn would help to explain the maintenance of genetic variation in natural populations and, in particular, the evolution and maintenance of sexual reproduction via Red Queen dynamics (Hamilton et al. 1990; Lively and Dybdahl 2000).

All hosts show specific and nonspecific responses, but the relationship between these two major components of the immune system has rarely been investigated, nor have the two fields of investigation—the analysis of defense costs and the analysis of specificity—had much interaction, either in theory or empirical research (Fellowes et al. 1998; Webster and Woolhouse 1998; Frank 2000; Jokela et al. 2000). It therefore still remains unclear how, in the sense of defense strategies and possible trade-offs, these two kinds of defenses interact and how the two fields might eventually be integrated. In this study, we experimentally tested the relationship between the two aspects of immune defenses in workers of the bumblebee *Bombus terrestris*. Encapsulation, the formation of a capsule consisting of melanized hemocytes surrounding the parasite (Söderhäll and Cerenius 1998), is a characteristic general, nonspecific constitutive response of invertebrates that can indiscriminately respond to a very wide range of antigenic challenges (Gupta 1986). The strength of encapsulation and melanization can be measured using standard methods (König and Schmid-Hempel 1995; Schmid-Hempel and Schmid-Hempel 1998).

Previous studies with *B. terrestris* have shown that host workers specifically resist different strains of the common, intestinal trypanosome parasite, *Crithidia bombi* (Trypanosmatidae, Zoomastigophorea [Lipa and Triggiani 1980] Schmid-Hempel et al. 1999; Brown et al. 2000). Although the underlying molecular processes are still unknown, these specific host-parasite interactions can easily be quantified by the variation in prevalence and intensity of infections by *C. bombi* (Schmid-Hempel and Schmid-Hempel 1993 Schmid-Hempel et al. 1999). In particular, *B. terrestris* hosts vary in the diversity of different parasite strains to which they are susceptible, with a given host line susceptible to a smaller or larger diversity of parasite strains. In this system, susceptibility to a larger diversity of strains also correlates with higher infection intensities (Schmid-Hempel et al. 1999) and thus with generally lower host fitness (M. Brown, R. Schmid-Hempel, and P. Schmid-Hempel, unpubl. ms.). Coinfections by several strains of *C. bombi* are regularly found in field colonies (P. Schmid-Hempel, unpubl. data).

In this study we tested whether hosts susceptible to only a few parasite strains (i.e., mounting a highly specific response) also are able to mount a strong (nonspecific) encapsulation response. This would be expected if, for example, a strong nonspecific immune response reflected overall immunocompetence. Alternatively, specific defense against parasite strains could come at the price of reduced nonspecific immunity as expected if some kind of trade-off between these two arms of the immune system were involved. To understand

TABLE 1. Mean encapsulation of an implant (arbitrary units, see text) and the mean cell count (per 200 μl of ringer solution) for each strain × colony interaction. Values in parentheses are standard deviations. Dashes represent subsamples for which there is only one replicate so a standard deviation could not be calculated.

Colony	Encapsulation	Strain 1	Strain 2	Strain 3	Strain 4
502	9.51	22.00	0	81.00	15.25
	(4.47)	(1.41)	(0)	(117.69)	(30.50)
536	11.65	46.33	0	67.33	15.50
	(6.02)	(39.80)	(0)	(40.15)	(23.16)
553	9.87	56	0	109.00	3
	(1.71)	(0)	(–)	(154.15)	(–)
562	14.30	88.25	4	147.25	84.40
	(2.35)	(116.20)	(7.38)	(210.68)	(143.49)
564	11.41	36.80	0	184.00	323.67
	(3.91)	(51.96)	(0)	(98.07)	(423.60)
574	7.43	0	3	33.0	122.00
	(0.71)	(–)	(–)	(44.03)	(50.91)
585	12.78	87.80	26.33	36.00	97.00
	(7.03)	(48.85)	(23.46)	(50.91)	(107.72)
607	9.78	22.00	0	148.50	19.33
	(2.99)	(49.19)	(–)	(95.39)	(68.08)
704	6.93	27.00	214.33	39.00	30.33
	(4.99)	(15.56)	(195.54)	(33.94)	(27.32)

these relationships offers novel insights into how immune defenses might be organized.

MATERIALS AND METHODS

Mated queens were collected in the spring of 2001 in northwestern Switzerland and allowed to rear colonies in the laboratory. Daughter queens of these lab colonies were mated and the resultant colonies were used in the experiment. All parasite strains used also originated from this location. No individual animals were reused for multiple assays.

Encapsulation Response

The assay followed previously established methods and yielded a repeatable measure of encapsulation (König and Schmid-Hempel 1995; Allander and Schmid-Hempel 2000). As workers eclosed they were taken from the colony and kept individually in boxes until they were five days old and ready to be used. Each worker received an implant (nylon fishing line 0.16mm × 0.8mm) inserted between the second and third sternite. The bees were then returned to their boxes. In none of the treatments were treated workers returned to the colony. The implant was removed two hours after implantation, the piece of nylon embedded in Eukitt (Electron Microscopy Sciences, Fort Washington, PA) and its degree of encapsulation/melanization were measured spectroscopically in arbitrary gray value units; higher values indicating stronger response (König and Schmid-Hempel 1995). On average, five workers were used. The mean encapsulation response was calculated for each colony used.

Infection with Crithidia

In the experiment, we infected a sample of workers (on average, three individuals per parasite strain) from each of several bumblebee colonies (thus, representing different host lines) with different strains of *C. bombi*. To prepare *C. bombi* strains, feces were collected from workers of naturally infected colonies, originating from the same locality as the test colonies (all test colonies were naive), and mixed with sugar water to create a standardized dose of 10,000 *Crithidia* cells per 20 μl of inoculum. Previous studies had shown that such inocula, prepared from different colonies, are genotypically different (P. Schmid-Hempel and C. Reber, unpubl. data) and generate specific responses in novel hosts (Schmid-Hempel et al. 1999). Each of the strains used here was checked and differentiated from the others by microsatellite analysis (data not shown; for methods, see Schmid-Hempel et al. 1999). We then characterized, for each colony, the susceptibility of its workers against the entire set of four *C. bombi* strains by separately measuring the infection intensities (cell counts) resulting from infections with each strain. For this, each strain was infected into, on average, three different individuals from a colony, and the response to this strain was estimated by the average infection intensity from these three measurements. Workers were four days old at the time of infection. Prior to infection, workers were starved for two hours and then fed the strain-specific inoculum. The workers were then kept for a week separately (Schmid-Hempel and Schmid-Hempel 1993). The intensity of infection was simply the number of *C. bombi* cells in gut (cells per whole gut plus 200 μl of Ringer's solution) of the infected workers. Finally, for the purpose of characterizing specificity (or narrowness of the response profile), we used the standard deviation and mean, and calculated the coefficient of variation for the average cell counts (from the infection by the four strains) for each colony.

Statistical Analyses

All analyses were done with SPSS 10 for the Macintosh (SPSS Inc., Chicago, IL). Standard deviations of the colonies' coefficients of variation were calculated from a bootstrapped (1000 iterations) sample drawn from the original data (Efron and Tibshirani 1993).

RESULTS

A total of 308 bees from 23 colonies were used in this experiment. Of these, nine colonies (179 bees) could be assessed for both their encapsulation response and specific susceptibility toward a total of four different lines of the parasite *C. bombi*. Table 1 shows, for each colony, the strength of the encapsulation response and the strain-specific cell counts (as a measure of resistance to this parasite strain). With respect to the latter, the infection experiments again confirmed the existence of a specific response against different strains of *C. bombi* (Schmid-Hempel 2001) for this sample of hosts. In particular, we found a highly significant colony-line x parasite-strain interaction for infection intensities (two-way analysis of variance; interaction term: $F_{57,180} = 1.64$, $P = 0.019$; partial $r^2 = 0.326$, data square-root transformed to normalize variances; strain: $F_{5,\ 72.320} = 3.124$, $P = 0.013$; colony: $F_{22,\ 69.694} = 1.303$, $P = 0.201$). We then characterized, for each colony, the susceptibility of its workers against the entire set of four *C. bombi* strains with the coefficient of variation. The coefficient of variation measure was found to

vary significantly over colony (Kruskal-Wallis: $\chi^2 = 4197.997$, $n = 9$, $P < 0.0001$). A low coefficient of variation indicates a low level of specificity in the response toward *C. bombi* (little variation in the response toward different strains; i.e., all strains can infect to a similar degree). A high coefficient of variation, in contrast, indicates a high specificity in the response to different strains (i.e., the response towards strains varies greatly; in the present case, only a few strains can infect). *Crithidia*, of course, would be fought by the nonspecific (general) immune response as well. However, any effect due to the general immune response would thus be equal for all strains in a single colony and would be ignored by the coefficient of variation (which measures the relative difference).

For each colony we also measured the non-specific immune response, that is, the average encapsulation response of its workers toward the general insult, the nylon implant. Similar to the specific response, we found that encapsulation varies significantly between colonies (data natural log(+1) transformed: one-way analysis of variance: $F_{10, 32} = 2.249$, $P = 0.04$).

When comparing these two measures for the specific and nonspecific arm of the immune system, respectively, we found that the strength of the nonspecific response correlates negatively with the ability to specifically resist a range of different parasite strains (Fig. 1; Spearman's rank correlation $r = -0.767$, $n = 9$, $P = 0.016$). We also calculated two other measures of variation (Zar 1996), Simpson's diversity index and Berger Parker's index in the place of the coefficient of variation, but obtained similar results (vs. encaps: Simpson's $r = 0.761$, $n = 9$, $P = 0.016$; Berger-Parker $r = 0.961$, $P < 0.0001$). This shows the overall result is not due to our choice of variation measure.

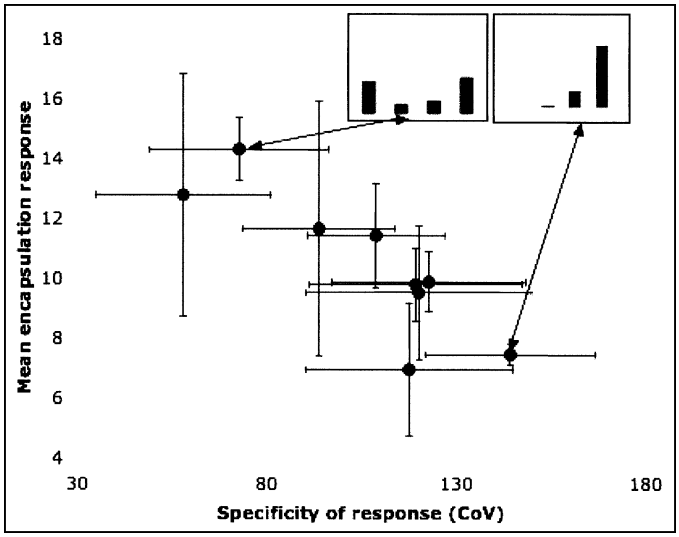

FIG. 1. The strength of the nonspecific response (degree of encapsulation; units) in relation to the susceptibility against an experimental range of different *Crithidia bombi* strains (measured as the coefficient of variation, CoV, of each colony's strain-specific cell counts, for infection intensity; high values indicate high degrees of specificity; i.e. exclusion of most parasites). Each point is the mean value for all tested workers of a given colony ($N \approx 20$ individuals per colony; see text). The relationship is for coefficient of variation versus encapsulation: Spearman's rank $r = -0.767$, $n = 9$ colonies, $P = 0.016$. The insets show the cell counts for that particular colony, each bar representing the percentage contribution of each of the four strains to the total observed infection intensity. The left inset is an example of an evenly distributed susceptibility to the four parasite strains; that is, low specificity. The right inset illustrates a specific response with an unevenly distributed susceptibility. The error bars represent the standard errors of the means calculated for the x-axis by bootstrapping the original population.

DISCUSSION

We set out to discover the relationship between the strength of the nonspecific immune response and the specificity of the specific immune response. Despite a relatively restricted sample for each within-colony and strain assay (imposed by the biological constraints of the system), we found that workers of a colony, with a good ability to defend themselves against parasites requiring a specific response, respond only weakly to a general insult (such as encapsulation of a novel antigen).

Natural selection may operate so that good quality hosts show a strong nonspecific encapsulation response and at the same time resist a wide range of parasite strains (i.e., are highly specific). Alternatively, the capacity to mount a highly specific response against many different parasite strains could compromise or even negate the need for the ability to deal with a generalized insult. Here we have evidence for the latter (Fig. 1). The results are consistent with predictions from theoretical models (Frank 2000) that seek to understand the combination of factors that favor either specific or nonspecific responses. The Frank model is a simple Lotka-Volterra system, in which hosts must compete for limited resources. Specific defense in the model is based on each host carrying a specific recognition allele; specific defense preventing an attack when the host shares the corresponding allele. Both types of defense carry costs for the host (as is the case for *B. terrestris*, see König and Schmid-Hempel 1995; Moret and Schmid-Hempel 2000) and there is a cost of virulence for the parasite. In his analysis, Frank found that hosts well protected by an array of specific responses should be selected to reduce the investment for the nonspecific immune response (and vice versa, in different circumstances, for high levels of nonspecific immunity).

It is important here to emphasize that we are not suggesting, for example, that highly specific colonies are fitter than those with a strong general immune arm, or vice versa. Rather the aim of the study was to elucidate the particular combination of specific and general immune abilities. Similarly, at present it is not known what kind of physiological mechanisms might be responsible for the observed inverse relationship (Fig. 1). Our experiments only suggest that hosts may not be capable of maintaining strong nonspecific responses and specifically resist many different strains of a parasite at the same time. Despite the incapacity to suggest any particular mechanism, the results shed a novel light on the possible constraints that affect the evolution of the immune system and particularly on the price that is paid for specificity. In fact, because we have measured the respective responses separately, in different individuals (but from the same host line), the negative relationship is not tied to the simultaneous activation of different components of the immune system within the same individual (that is, likely does

not reflect a kind of utilization cost) but rather might reflect an intrinsic, evolved element in the architecture of the immune system.

Acknowledgments

This study was financially supported by the Swiss National Science Foundation, Bundesamt fur Bildung und Wissenschaft and the European Union (Improving Human Potential fellowship for EBM). We thank Y. Moret for technical assistance and P. Korner, V. Müller, and S. Bonhoeffer for comments.

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