

Effect of temperature and relative humidity on the cellular defense response of *Ephestia kuehniella* larvae fed *Bacillus thuringiensis*

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Abstract

While maintained under all combinations of three temperatures and two RH, fifth instar larvae of the Mediterranean flour moth, *Ephestia kuehniella* were fed wheat treated with spores and crystals of *Bacillus thuringiensis* var. *kurstaki*. Larvae that had fed on wheat with the bacterial preparation contained higher concentrations of nodules in their haemocoel than did larvae fed on wheat without bacteria. Nodule concentrations in larvae fed untreated wheat were unaffected by temperature or relative humidity. Larvae fed treated wheat had higher nodule concentrations at 32 °C than at 15 and 23 °C, and higher nodule concentrations at a relative humidity of 85% than at 43%. The percentage of larvae that pupated was lower when larvae were fed the bacterial preparation, and was significantly higher at 23 °C than at 15 and 32 °C, regardless of whether larvae were fed bacteria or not. Less time was required for larvae to develop to pupation at higher temperatures and at higher humidity. Mean time to pupation was lower for bacteria-fed larvae than for those fed untreated wheat, and this appeared to be a result of truncation of the distribution of times to pupation because only rapidly developing larvae survived to pupation.

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1. Introduction

Insects are subject to the attack of pathogens and parasitoids. The insect integument and gut function as external barriers to prevent infection. If the external barriers are penetrated, specific haemocytes mediate cellular immunity, and induced peptides and proteins mediate humoral immunity (Gillespie et al., 1997). Cellular immunity is mediated by several types of haemocytes (Lavine and Strand, 2002), which mediate reactions such as phagocytosis, nodule formation, and encapsulation (Ratcliffe and Rowley, 1979). In general, nodulation is the response to objects smaller than blood cells, and in the later stages of nodule formation, melanization occurs (Gotz, 1986).

The interaction of pathogens with insects is affected by environmental conditions such as temperature and relative

humidity (RH). The most widely studied example of the influence of temperature is behavioral fever, in which infected or parasitized poikilotherms elevate their body temperature by altering thermoregulatory behavior (Ouedraogo et al., 2003). Behavioral fever favors insect host survival in a wide range of interactions including Orthoptera infected with protozoa, fungi or bacteria (Boorstein and Ewald, 1987; Bronstein and Conner, 1984; Bunday et al., 2003; Inglis et al., 1996; Ouedraogo et al., 2003), Diptera infected with fungi (Watson et al., 1993) and lepidopterous larvae parasitized by tachinid fly larvae (Karban, 1998). In contrast, pathogenic effects of *Bacillus* spp. on a wide range of insects are increased at elevated temperature (e.g., Glare and O'Callaghan, 2000; Katbeh-Bader et al., 1999; Li et al., 1995; Van Frankenhuyzen, 1994). High RH is associated with high mortality in hosts infected with fungal (Hajek and St. Leger, 1994; Luz and Fargues, 1999; Shipp et al., 2003) and viral (Fuxa et al., 1999) pathogens; however, effects on hosts infected with

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bacteria are variable (Affify and Matter, 1970). Environmental influences on host–pathogen interactions may operate through the biology of the pathogen (Arthurs and Thomas, 2001), the immune response of the host (Ouedraogo et al., 2003), or the rate of pathogen entry into the host (Katbeh-Bader et al., 1999).

The Mediterranean flour moth, *Ephestia kuehniella*, is a serious cosmopolitan pest of stored products, especially flour (Brindley, 1930). However, there have been few studies on the effect of bacterial infection on the mortality and immune response for this insect. *Bacillus thuringiensis* var. *thuringiensis* (*Btk*) was originally isolated from *E. kuehniella*, and Heimpel and Angus (1959) regarded the insect's response to the bacterium as unique, in that infected larvae do not exhibit general paralysis, and death occurs only if bacterial spore germination occurs in the mid-gut in the presence of the crystalline endotoxin. Following ingestion of droplets of spore-crystal suspension, larvae die within two days and bacterial spores proliferate in the cadaver (Takatsuka and Kunimi, 1998). Following exposure to lower concentrations of dietary *Btk*, larval *E. kuehniella* display elevated rates of melanization in haemolymph (Rahman et al., 2004).

There is no information on the influence of temperature and RH on the immune responses of insects when they are challenged with bacteria. The aim of this study is to examine the cellular immune response of *E. kuehniella* larvae fed on media mixed with *Btk*, and to determine the effect of temperature and RH on this response.

2. Materials and methods

2.1. Insects

Eggs of *E. kuehniella* were obtained from Beneficial Insectary, and reared in 4 L glass jars half filled with wheat (14% w/w moisture). Fifty milliliters of glycerol and 50 ml of honey were added to the top of the wheat and covered with a layer of partially ground wheat. The rearing conditions were 23 °C, 65% RH, and 16:8 h L:D photoperiod. The development of different larval instars was observed by detecting the moulted head capsules. Fifth instar larvae were used in experiments because of their high rate of feeding and weight gain (Brindley, 1930).

2.2. Temperature and relative humidity

Three temperatures: 15, 23, and 32 °C, and two RH: 43 and 85% were used. Temperatures were maintained using reach-in growth chambers. To produce the levels of RH, saturated solutions of potassium carbonate (43% RH) and potassium chloride (85% RH) (Solomon, 1951) were poured into plastic containers (30 cm length, 25 cm width, and 14 cm height) to a depth of 2 cm. One container of each solution was placed in each growth chamber, to produce the six combinations of temperature and RH used. A wooden rack with plastic screening supported the vials with insects above

the salt solutions. The plastic containers were sealed with a tightly fitting plastic cover; a small cotton-plugged hole in the cover allowed air exchange for respiration.

2.3. Bacteria

Safer's BTK was used for treating the wheat on which larvae were fed during the experiment. This formulation consists of both bacterial spores and crystal endotoxin and contains 12.7 million international U/ml *B. thuringiensis* var. *kurstaki*. Three liters of wheat were treated with 3.33 ml/L of bacterial formulation by pouring the formulation onto the wheat in a large container. The wheat was then thoroughly mixed by tumbling it in a closed container for 2 h.

2.4. Procedure

Thirty vials (7 cm height × 2.8 cm diameter) were each two-thirds filled with *Btk*-treated wheat (about 25 g per vial). An additional 30 vials were similarly filled with untreated wheat. Ten early fifth instar larvae of *E. kuehniella* were placed in each vial and, the vial closed with a ventilated plastic cap. Five vials of *Btk*-treated wheat and five vials of untreated wheat were placed in each of the six containers with regulated RH and temperature.

When 48 h had elapsed from the start of the experiment, three vials were removed from each treatment combination and 10 living larvae were randomly selected for examination of haemocyte nodulation. Larvae were anesthetized by placing them on ice, and then the dorsal blood vessel was punctured with an alcohol sterilized fine needle. The resulting drop of haemolymph was placed in an American Optical Bright-Line haemocytometer, and all melanized dark nodules in the haemocytometer cell were counted under a stereomicroscope at 400×. Counts were converted to concentrations of nodules/μl of haemolymph by dividing by the total haemocytometer cell volume (1.089 μl). Every day for 15 days after set-up, the remaining two vials from each treatment combination, initially containing 20 larvae, were examined to record mortality and time to pupation.

2.5. Statistical analysis

Data on nodule concentration and time to pupation were subjected to analysis of variance. Nodule concentration was square root transformed to stabilize variance before analysis. Effect of treatments on frequency of larvae pupating was examined using log linear analysis of contingency tables (Bishop et al., 1975), in which all main factors and all interactions of frequency of pupation were included in the model. Preliminary analyses indicated treatment responses of nodule concentration and frequency of pupation were markedly different in the presence and absence of the *Btk* treatment. To elucidate these responses, overall analyses were followed by separate analyses on larvae receiving each treatment. Following the separate analyses

of variance, Tukey’s pairwise comparisons of all means were carried out, where appropriate. All analyses were performed using Systat 10.2 (Systat, 2002).

3. Results

The concentration of nodules in haemolymph (Table 1) was affected by feeding treatment, RH and temperature (Table 2), as well as the interaction of feeding treatment with the environmental conditions. The mean concentration of nodules for larvae fed untreated wheat was $0.20 \pm 0.05/\mu\text{l}$, and was unaffected by environmental conditions (Table 2). The effects of temperature and RH on nod-

ule concentration were restricted to larvae fed the *Btk* preparation. Within the *Btk*-fed larvae, nodule concentration averaged $46 \pm 4/\mu\text{l}$ at 32 °C, which was significantly higher than at 23 °C ($28 \pm 4/\mu\text{l}$), and 15 °C ($27 \pm 5/\mu\text{l}$). At 85% RH, nodule concentration in *Btk*-fed larvae averaged $43 \pm 4/\mu\text{l}$, which was greater than the average concentration of $24 \pm 3/\mu\text{l}$ at 43% RH. Although the concentration of nodules in *Btk*-fed larvae was not significantly influenced by the interaction of temperature and RH (Table 2), the observed pattern of nodule concentrations in bacteria-fed larvae showed a tendency for the highest concentrations to occur at 85% RH or at either humidity at the highest temperature (Table 1).

Table 1

Effect of feeding *B. thuringiensis*-treated wheat to fifth instar *E. kuehniella* larvae at different temperatures and RH on the concentration of nodules in haemolymph, duration of development to pupation, and percentage of larvae pupating

Treatment	Measure	Conditions					
		15 °C		23 °C		32 °C	
		43% RH	85% RH	43% RH	85% RH	43% RH	85% RH
<i>B. thuringiensis</i>	Nodules/ μl (mean \pm SEM)	13.2 \pm 3.2a	39.9 \pm 7.1c	18.4 \pm 4.4ab	36.9 \pm 5.0bc	41.4 \pm 4.8c	50.9 \pm 6.0c
	Days to pupation (mean \pm SEM)	7.9 \pm 1.0	5.0 \pm 0.9	5.3 \pm 0.4	4.1 \pm 0.4	4.0 \pm 0.5	3.2 \pm 0.3
	Pupation (%)	45	25	80	45	25	30
Untreated	Nodules/ μl (mean \pm SEM)	0.4 \pm 0.2a	0.3 \pm 0.1a	0.1 \pm 0.1a	0.1 \pm 0.1a	0.1 \pm 0.1a	0.3 \pm 0.1a
	Days to pupation (mean \pm SEM)	8.2 \pm 0.8	6.7 \pm 0.6	6.8 \pm 0.6	4.3 \pm 0.4	4.8 \pm 0.3	3.6 \pm 0.3
	Pupation (%)	55	70	90	95	80	75

Means of nodule concentrations followed by the same letter do not differ significantly within rows (Tukey’s comparison of all means within each feeding treatment with $\alpha = 0.05$).

Table 2

Results of analysis of variance of nodule concentrations (after square root transformation), log-linear analysis of contingency tables of frequency of pupation, and analysis of variance of days to pupation

Treatment	Nodule concentration				Frequency of pupation			Days to pupation			
	df	SS	F	P	df	LR χ^2	P	df	SS	F	P
Analysis over both feeding treatments											
<i>Btk</i>	1	598.736	546.2	<0.001	1	33.30	<0.001	1	19.450	5.6	0.02
Temp	2	21.656	9.9	<0.001	2	16.95	<0.001	2	157.557	22.5	<0.001
RH	1	23.601	21.5	<0.001	1	0.72	0.40	1	80.825	23.1	<0.001
<i>Btk</i> *Temp	2	22.590	10.3	<0.001	2	2.62	0.27	2	0.602	0.1	0.92
<i>Btk</i> *RH	1	22.923	20.9	<0.001	1	2.97	0.09	1	0.089	0.0	0.87
Temp*RH	2	4.446	2.0	0.14	2	1.16	0.56	2	5.854	0.8	0.44
<i>Btk</i> *Temp*RH	2	5.623	2.6	0.08	2	3.40	0.18	2	10.362	1.5	0.23
Error	108	118.385						131	458.937		
Analysis of <i>Btk</i> -fed larvae											
Temp	2	44.153	10.2	<0.001	2	11.60	0.003				
RH	1	46.522	21.5	<0.001	1	3.81	0.05				
Temp*RH	2	10.034	2.3	0.11	2	3.47	0.18				
Error	54	117.007									
Analysis of larvae fed untreated wheat											
Temp	2	0.094	1.8	0.17	2	11.11	0.004				
RH	1	0.002	0.1	0.76	1	0.47	0.49				
Temp*RH	2	0.035	0.7	0.51	2	1.00	0.61				
Error	54	1.379									

The analyses of nodule concentration and frequency of pupation were initially performed over both *Btk*-treated and untreated wheat, and then separately for each treatment. Treatments were *Btk* (the presence or absence of *Bacillus thuringiensis* var. *kustaki* in the wheat diet), Temp (temperature), and RH (relative humidity). Analyses of variance are of complete models. Treatment designators in the log-linear analysis signify the interaction of the treatment term with “pupation” (the frequency of pupation or death of larvae), in models that included all main factors and their interactions with pupation.

All larvae in the experiment either successfully pupated, or died. In the analysis over both feeding treatments, the proportion of larvae pupating (Table 1) was affected by whether or not they had been fed the *Btk* preparation (Table 2). Over all temperature and RH conditions, 78% of larvae fed untreated wheat successfully pupated but only 42% of *Btk*-fed larvae pupated. Temperature also affected pupation success, which was greatest at 23 °C and lower at 15 and 32 °C. Although interactions of feeding treatment with environmental conditions were not significant in the overall analysis, analyses within the *Btk* treatment did reveal different patterns of pupation success. In bacterial treatments, pupation success was influenced by temperature (Table 2) and tended to be highest at 23 °C. RH did not influence survival of larvae fed the untreated wheat. However, in *Btk*-fed larvae RH had a significant effect (Table 2), and the most noticeable feature of the data is that pupation success was much higher at 23 °C and 43% RH than in any other combination of environmental conditions (Table 1). In the *Btk* treatment, pupation success tended to be lowest in conditions where nodule concentration was highest, although the correlation of the two was not significant ($r = -0.71$, $n = 6$, $P \approx 0.12$).

Pupation success affected sample size in the analysis of duration of larval development to pupation, but nevertheless the overall analysis appeared robust as the minimum group size was 39 among significantly differing treatment groups. Separate analyses for each feeding treatment were

not performed because of concerns about variable and small sample size. In the overall analysis, the time required to develop to pupation (Table 1) was significantly influenced by temperature (Table 2), and averaged 7.2 ± 0.4 days at 15 °C, 5.2 ± 0.3 days at 23 °C, and 4.0 ± 0.2 days at 32 °C. RH also influenced time to pupation: pooled over other treatments, time to pupation was 6.2 ± 0.3 days at 43% RH and 4.6 ± 0.2 days at 85% RH. The mean time to pupation of *Btk*-fed larvae (5.1 ± 0.3 days) differed significantly from that for larvae fed untreated wheat (5.6 ± 0.3 days). For each combination of environmental conditions, the mean time to pupation was consistently lower for the *Btk* treatment than for the corresponding untreated control (Table 1). The minimum time to pupation for the *Btk* treatment and for the control was usually the same for each combination of environmental conditions (Fig. 1). However, the maximum time to pupation was usually less in the *Btk* treatment than in the corresponding control (Fig. 1). Thus, the *Btk* treatment did not induce more rapid larval development, but rather the reduced mean time to pupation in the *Btk* treatment resulted from truncation of the right hand tail of the distribution of time to pupation. In the *Btk* treatment, larvae with lower development rates died rather than pupated.

4. Discussion

Ephestia kuehniella fed untreated wheat survived best at moderate temperatures; optimal conditions for survival in our study were similar to those optimal for growth reported by Jacob and Cox (1977). The reduced survival of untreated larvae at 32 °C may be symptomatic of heat stress, as Bell (1975) reported that *E. kuehniella* larvae reared at 30 °C produce sterile adults, and at 31 °C fail to pupate. In our study, *Btk*-fed larvae survived best at the intermediate temperature, and survival was poor at 32 °C. Many serovars of *Bt* are more toxic at higher environmental temperatures (Glare and O'Callaghan, 2000). High temperatures favour more rapid bacterial reproduction (Van Frankenhuyzen, 1994) and may increase dietary intake of pathogens (Ferro and Lyon, 1991; Katbeh-Bader et al., 1999). The poor survival of *Btk*-fed larvae we observed at 15 °C compared with 23 °C was unexpected: generally, *Bt* has diminished toxicity at 15 °C and below (Glare and O'Callaghan, 2000).

The shorter time to pupation at high humidity that we observed is in accord with Afify and Matter (1970), who noted slower development at low humidity. However, our finding of higher mortality at high humidity in the *Btk* treatment is opposite to the findings of earlier studies in which *E. kuniella* were exposed to *Btk* throughout larval development and mortality was highest at low humidity (Afify and Matter, 1970; Gibson and Wolf, 1962). Afify and Matter (1970) suggested that their results were attributable to slower development at low humidity, resulting in prolonged exposure of the more vulnerable early instars to *Btk*. Such an effect would not have been evident in our study, which used only fifth instars.

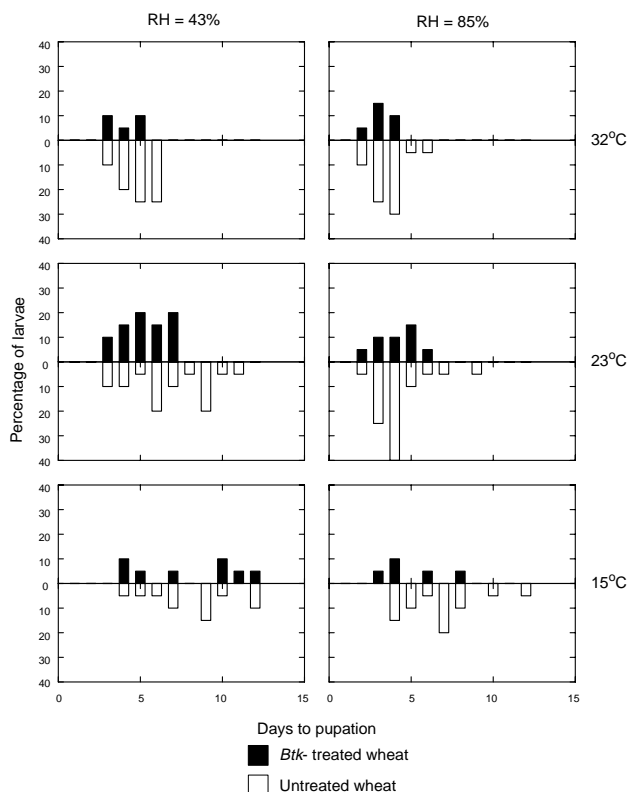


Fig. 1. Effect of feeding of *B. thuringiensis* on the frequency distribution of days to pupation of *E. kuehniella* larvae under six combinations of temperature and relative humidity.

We observed lower concentrations of nodules than seen in studies in which bacteria were injected into the insect (Bedick et al., 2000; Diehl-Jones, 1987; Miller et al., 1994, 1996; Stanley et al., 1991; Tunaz et al., 1999). Injection allows fast and direct interaction between the haemocytes responsible for the immune response and the stimulating bacteria. Thus, the lower nodule concentration we observed could be a characteristic of insects infected through feeding. Even though the site of action of *Btk* is the mid-gut epithelium, infection from feeding is almost immediately accompanied by changes in haemolymph, probably due to leakage of gut contents or other elicitors into the haemocoel (Heimpel and Angus, 1959; Rahman et al., 2004). Presumably the nodulation we observed was in response to this leakage. Perhaps this unusual mechanism of induction of the cellular immune system accounts for the atypical temperature response in which insects fed *Bacillus* spp. suffer more mortality at higher temperature. The reduced mortality at elevated temperatures of insects infected with other pathogens is at least partly attributable to immune responses (Bundey et al., 2003; Ouedraogo et al., 2003) and typically involves pathogens immersed in the haemocoel (Bronstein and Conner, 1984; Bundey et al., 2003; Karban, 1998; Ouedraogo et al., 2003; Watson et al., 1993). A comparison of temperature dependence of immune responses of *E. kuehniella* larvae to dietary and injected *Btk* could be used to test this hypothesis.

Cellular immune responses of *E. kuehniella* fed low doses of *Btk* have previously been inferred, based on elevated rates of melanization (Rahman et al., 2004). In our study, immune responses appeared to be generally insufficient to prevent larval death at the *Btk* dose we used, as the *Btk*-fed treatments with high nodule concentrations also tended to be those with highest mortality. However, Rahman et al. (2004) found that immune responses in *E. kuehniella* larvae are correlated with tolerance to *Btk*, are transmissible to offspring by a maternal effect, and are a possible mechanism for resistance to *Btk*. Their work was carried out at 25 °C and unspecified RH. Our study demonstrated temperature- and humidity dependence of immune responses. Consequently, future investigations aimed at relating immune responses of *E. kuehniella* to resistance development under the variable conditions of operational *Btk* applications should involve multiple combinations of temperature and humidity.

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