LABORATORY STUDY OF THE INFLUENCE OF WATER TEMPERATURE AND PH ON *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* EFFICACY AGAINST BLACK FLY LARVAE (DIPTERA: SIMULIIDAE)

J. O. LACOURSIÈRE[1] AND G. CHARPENTIER

Groupe de Recherches sur les Insectes Piqueurs, Département de Chimie-Biologie, Université du Québec à Trois-Rivières, Trois-Rivières, Québec, Canada G9A 5H7

ABSTRACT. An experimental formulation of *Bacillus thuringiensis* var. *israelensis* was used in the laboratory to assess the influence of water temperature and pH on the relationship between concentration, duration of exposure, and mortality of the northern black fly species *Simulium decorum* and *Prosimulium mixtum/fuscum* group. Mortality increases in both species with increases in duration of exposure, concentration, temperature and pH. Onset of death is shortened by increase in concentration and temperature. As temperature rises, the concentration of *B.t.i.* required to induce mortality decreases; the sharpest decline occurring between 12 and 18°C for *S. decorum*, and between 4 and 8°C for *P. mixtum/fuscum* larvae. Lower pH induces a loss of efficacy of the *B.t.i.* formulation on *S. decorum* larvae at 4 and 12°C. Dialysis of the *B.t.i.* formulation at pH 11 for 2 h increases its potency against *S. decorum* larvae, suggesting an effect of an extralarval alkaline hydrolysis on the *B.t.i.* efficacy. An alkaline prehydrolysis of the paracrystalline bodies could therefore be used in cold and acidic environments to compensate for loss of efficacy.

INTRODUCTION

Black flies (Diptera: Simuliidae) are serious pests in many parts of the world because of prodigious blood-feeding, injection of toxic saliva into wounds, and transmission of pathogens and parasites to man and other homeothermic animals. In some areas, particularly in West Africa, the sanitary problems for humans are severe enough to warrant massive control actions. Although black flies are not generally incriminated as specific diseases vectors for humans in Canada, their attacks can be severe enough to restrict outdoor activity. Pathogen transmission, reduction in productivity, as well as mortality have been documented in livestock and wildlife (National Research Council Canada 1982). The increasing importance of natural resources and the need to develop and exploit them, brought necessity for control programs ensuring that the productivity and well-being of the workers is not eroded.

Chemical pesticides such as DDT, methoxychlor, chlorphoxim and temephos have traditionally been used to control black fly larvae, but such measures are becoming less effective as larval resistance and environmental safety concerns develop (Kurtak 1986). Low temperatures encountered in northern latitudes are also a limiting factor to the efficacy of chemical insecticides such as temephos (Back et al. 1979). In the search for a safe, economical black fly larvicide, the entomopathogen *Bacillus thuringiensis* var. *israelensis* (Goldberg and Margalit 1977) has rapidly gained acceptance as the most promising alternative to chemical larvicides because of its efficacy, selectivity, biodegradable nature, and long shelf life (Lacey 1985). The potential of *B.t.i.* as a black fly larval control agent has been investigated in the laboratory and in the field since 1978 (Gaugler and Finney 1982). Most research has been directed toward use at lower latitudes on such serious human pests as *S. damnosum s. l.* The relationship between concentration, duration of exposure, and effectiveness of different *B.t.i.* formulations has already been reviewed (Gaugler and Finney 1982). However, relatively few workers have investigated the effect of temperature as a limiting factor to effectiveness (Lacey and Federici 1979, Molloy et al. 1981, Colbo and O'Brien 1984, Back et al. 1985). Very little is known about the influence of water pH on *B.t.i.* potency (Lacey et al. 1978, Sinègre et al. 1980, Ignoffo et al. 1981).

In this study we assessed the influence of water temperature and pH on the relationship between concentration, duration of exposure and efficacy of the *B.t.i.* protoxin on some Canadian black fly species.

MATERIALS AND METHODS

Laboratory experiments were conducted between the summer 1981 and winter 1984 on larvae of *Simulium decorum* Walker and *Prosimulium mixtum/fuscum* Syme and Davies group. *Simulium decorum* is widely distributed

[1] Current address: Department of Entomology, University of Alberta, Edmonton, Alberta, Canada T6G 2E3.

over most of North America. Immatures of this species are typical of warm, food-rich outflows originating from lakes, impoundments, and beaver ponds, having been collected in water temperatures ranging from 4 to 31°C. Females feed on wild and domestic animals, as well as on humans, and are regarded as a nuisance in some regions (Adler and Kim 1986, Currie 1986). Members of the *Prosimulium mixtum/fuscum* group are encountered from northern Saskatchewan, east to Newfoundland, and south to West Virginia. Immatures of this group overwinter as larvae, and are usually collected in streams with temperatures from 0 to 14°C. Females of this species can also be pests in the spring (Adler and Kim 1986, Currie 1986).

Larvae of *Simulium decorum* and *Prosimulium mixtum/fuscum* were collected from two sites in the Trois-Rivières area (Québec, Canada): streams in the St-Thimothé d'Hérouxville municipality (46° 44′ N; 72° 44′ W) and the Lac des Souris outlet (46° 35′ N; 72° 58′ W). Larvae were maintained under laboratory conditions in a short-term maintenance system (Lacoursière and Boisvert 1987) until the start of the experiment. Mixed instars (4 to 6) of a 90–95% pure population were used in each trial.

An experimental preparation of *Bacillus thuringiensis* var. *israelensis* (TEKNAR WDC, Zoecon Industries Limited, U.S.A.) (formulation titer of 1.27×10^7 viable spores/mg) was used in this study. Manufacturer specifications listed an activity of 600 *Aedes aegypti* International Toxic Units (ITU)/mg.

Solutions for pH effect experiments were prepared as follows: pH 4.5 was obtained by adding HCl (0.1 M) to a Na_2HPO_4 (10 mM) solution; pH 7 by mixing adequate proportions of Na_2HPO_4 and NaH_2PO_4 (10 mM) solutions; pH 9 by the addition of NaOH (0.1 M) to a Na_2HPO_4 (10 mM) solution.

Dialysis of the *B.t.i.* inoculum was effected with cellulose dialyzing tubing (12,000 MW) submerged in an agitated solution of sodium carbonate at pH 11 (Na_2CO_3 10 mM + NaOH 0.1 M). The first set of control beakers (pH 7) received a 0.8 ml inoculum of solution (pH 11), while the second set received 0.8 ml (400 ppm) of dialysate (*B.t.i.* formulation dialysed 2 h).

The bioassay procedure used was a modified version of the "Jar tests" technique (Jamnback 1973). Beakers were filled with 500 ml of distilled water (pH 6.8), or with prepared solutions for the water pH influence study. Flow was induced by passing compressed air through diffusers, and temperature was maintained at 4, 8, 12 and 18 ± 0.2°C using thermoregulated baths (Haake F3C, Fisher Inc., U.S.A.) (Lacoursière 1984[2]). A minimum of 50 larvae were placed in each beaker, and five replicates of each exposure duration and concentration combination were performed per experiment. Three beakers were always left untreated and used as controls. After a minimum 12 h pre-exposure acclimatization period without any food, a calibrated amount of an aqueous suspension of *B.t.i.* was added to the beakers. No food was provided during the experiment. At the end of the exposure period, the inoculum was removed (i.e., each beaker was rapidly inverted over a sieve, rinsed twice and refilled with distilled water or a prepared solution kept at experimental temperature). If any live or dead larvae drifted during the inversion phase, they were immediately placed back into the beaker. The samples were observed at 2, 4, 8, 12 and 24 h of the experiment. Larvae that were presumed dead were probed for possible movement with the rounded tip of a Pasteur pipet and data on the number of dead and live larvae in each beaker were recorded. Dead larvae were preserved in 95% alcohol for identification.

Larvae of both species were exposed for 15 and 30 min, 1, 2, 3 and 4 h to a concentration of 400 ppm of *B.t.i.* (TEKNAR WDC) at temperatures of 4, 8, 12 and 18°C in the first bioassay trial, while exposed for 30 min to concentrations of 0.4, 4, 40, 200 and 400 ppm in the second. In a third trial, *Simulium decorum* larvae were exposed 30 min to 400 ppm of *B.t.i.* in solutions of pH 4.5, 7 and 9 at temperatures of 4 and 12°C. In a fourth experiment, larvae of *Simulium decorum* were exposed 30 min to 400 ppm of a dialyzed *B.t.i.* inoculum (2h at pH 11). Neither larval metabolites nor *B.t.i.* inoculum modified the pH of the solution during the experiment. Mortality in control beakers was under 3% for all experiments. Because of heterogeneity of variance in some samples, the nonparametric multisample Kruskal-Wallis test on mortality was used. The LC_{50} and LC_{90} were estimated using a Log-Probit Analysis.

RESULTS AND DISCUSSION

The progression in mortality of *Simulium decorum* and *Prosimulium mixtum/fuscum* larvae induced by an experimental preparation of *Bacillus thuringiensis* serovariety *israelensis*

[2] Lacoursière, J. O. 1984. Étude des principaux facteurs influençant la pathogénécité de *Bacillus thuringiensis* sérovariété *israelensis* envers les larves de mouches noires (Diptères: Simuliidae). M.Sc. thesis, Université du Québec à Trois-Rivières, Trois-Rivières, Québec, Canada. 191 pp.

(TEKNAR WDC) is shown in figures 1, 2 and 3. For a concentration of 400 ppm, the increases in mortality of both species is maximum when the exposure is lengthened between 15 and 60 min; this optimal exposure duration is reduced by an increase in temperature (figures 1 and 3). Little or no significant additional increase ($P \leq 0.05$) in total mortality of *S. decorum* larvae is observed when the exposure is lengthened over 3 h at 4 and 8°C, and 30 min at 12°C. Likewise exposure exceeding 3 h at 4°C, 2 h at 8°C, and 60 min at 12°C had no additional effect on total mortality of *P. mixtum/fuscum* larvae. Potency tests of the *B.t.i.* solutions at the end of the different exposure periods, done on *Aedes triseriatus* (Say), did not show any significant decline in the initial concentration of *B.t.i.* (400 mg/liter) with respect to increasing exposure duration. Moreover, because of the relatively high concentration used, decline in concentration due to removal of *B.t.i.* by larval feeding and/or adsorption on the different surfaces would be

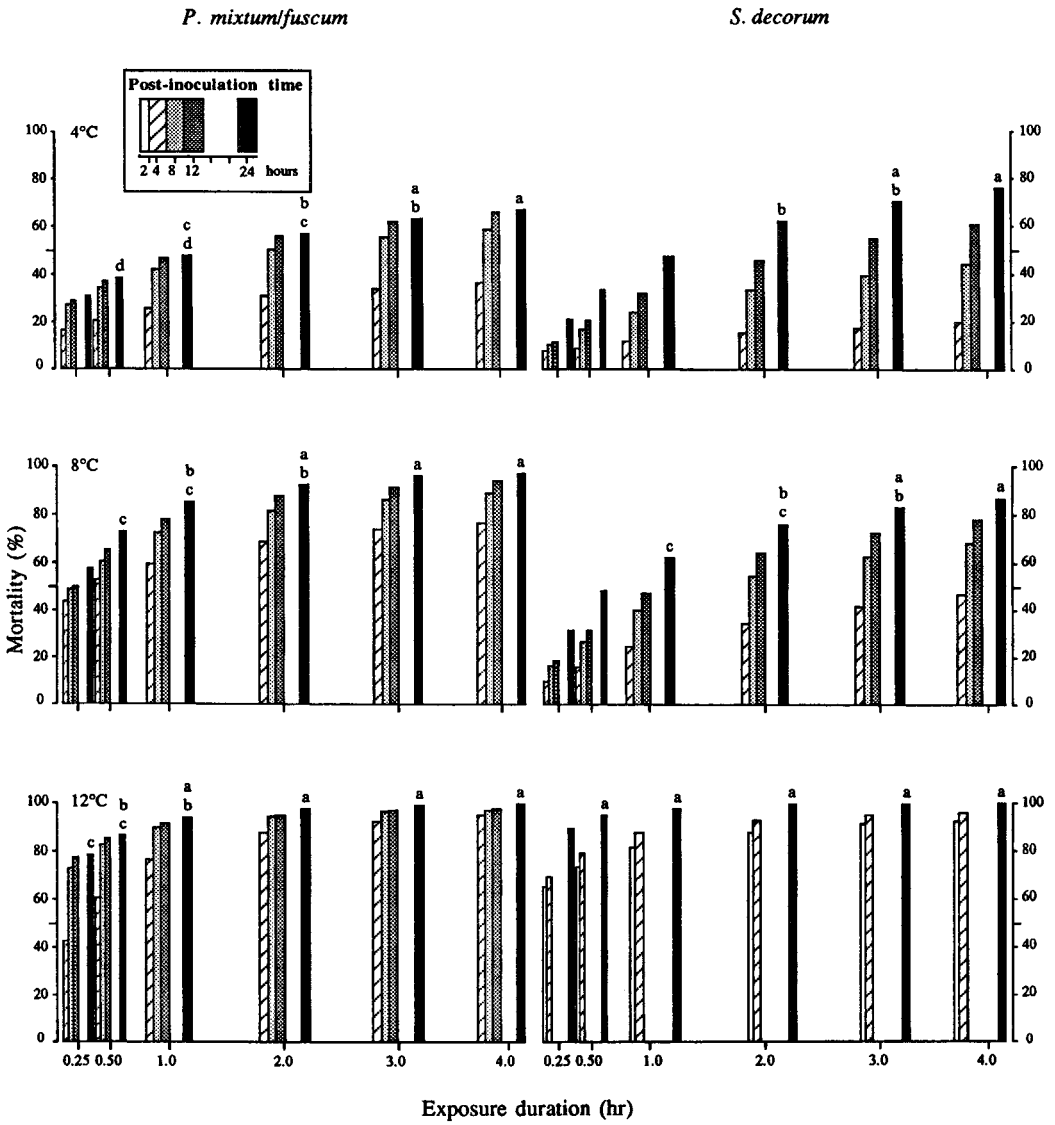

Fig. 1. Effect of exposure duration on *Bacillus thuringiensis* var. *israelensis* (TEKNAR WDC) efficacy on larvae of **A)** *Simulium decorum*, and **B)** *Prosimulium mixtum/fuscum* group, for a concentration of 400 ppm (Total mortality means crowned with the same letter are not significantly different at 5% error using a Kruskal-Wallis test).

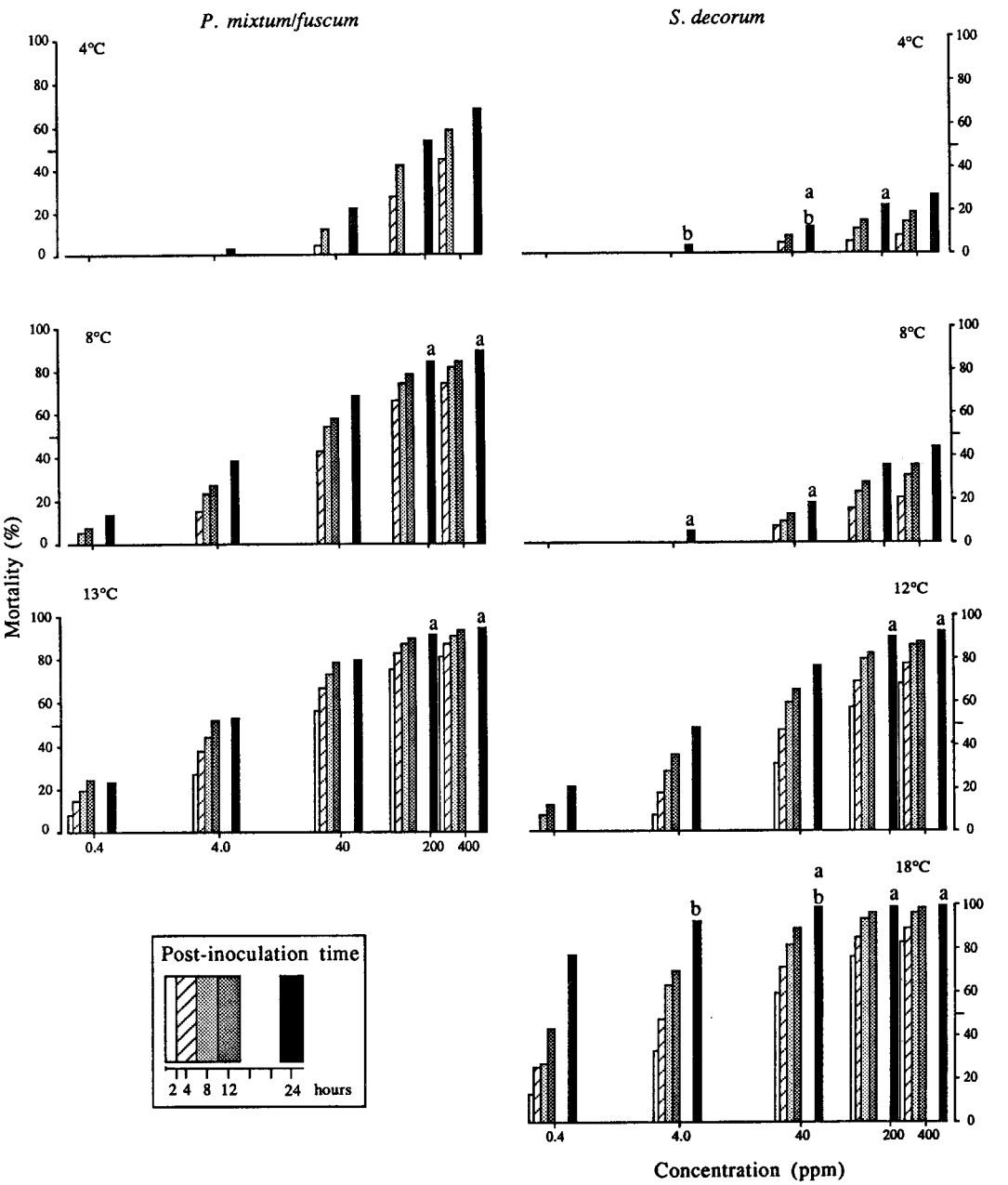

Fig. 2. Effect of concentration variation on *Bacillus thuringiensis* var. *israelensis* (TEKNAR WDC) efficacy on larvae of **A)** *Simulium decorum*, and **B)** *Prosimulium mixtum/fuscum* group, for an exposure duration of 30 min (Total mortality means crowned with the same letter are not significantly different at 5% error using a Kruskal-Wallis test).

unlikely to have any significant bearing on the relation between exposure duration and mortality. For an exposure duration of 30 min, increase in *B.t.i.* concentration induced an increase in the mortality of both species; the magnitude of the increase depends on the temperature and the species (Figs. 2 and 3). Only a substantial increase (≈200 ppm) in concentration over a minimum of 4 ppm induced a significant increase in total mortality of *S. decorum*

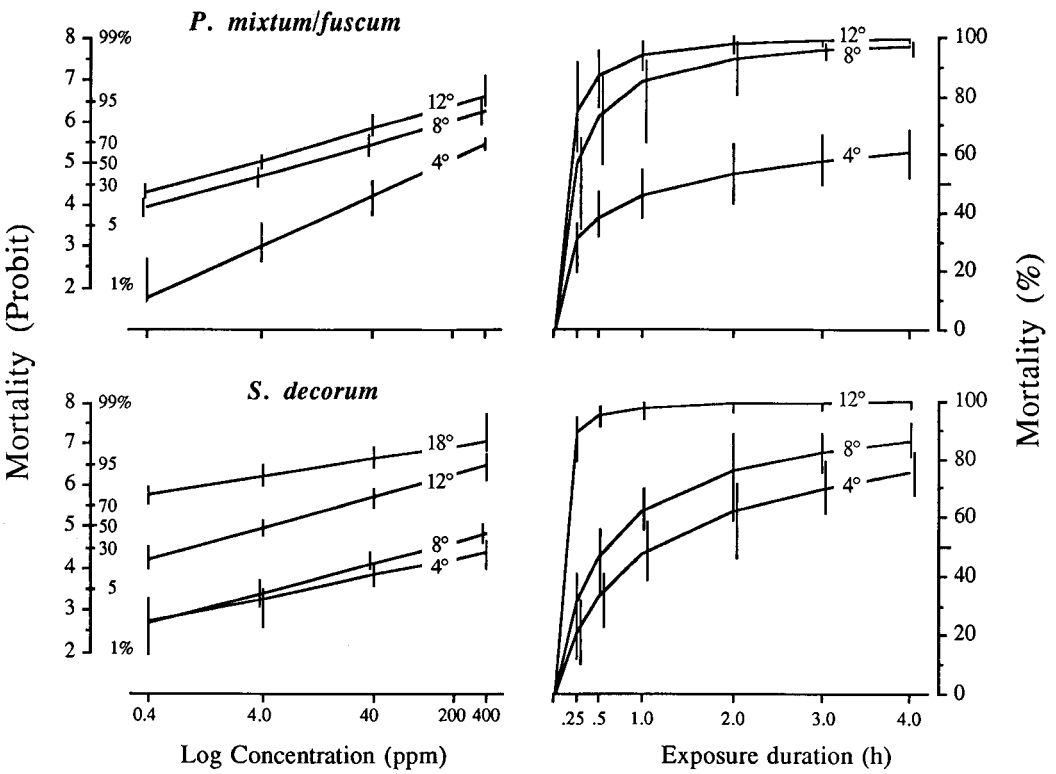

Fig. 3. Effect of temperature on *Bacillus thuringiensis* var. *israelensis* (TEKNAR WDC) efficacy on larvae of *Simulium decorum* and *Prosimulium mixtum/fuscum* group (95% confidence limit).

larvae at 4 and 8°C, while an increase exceeding 200 ppm at 12°, and 4 ppm at 18°C induced no additional larvicidal effect. Under the same conditions, an increase in concentration from a minimum of 4 ppm at 4°C induced a mortality increase in *P. mixtum/fuscum* larvae, while an increase over 200 ppm produced no significant additional effect on the final mortality at 8 and 13°C. These results corroborate those of Lacey and Federici (1979) and Frommer et al. (1980) who observed an increase in total mortality of *S. vittatum* Zetterstedt larvae as exposure and concentration increased. Lacey and Federici (1979) observed higher mortalities when exposure to 10 ppm of *B.t.* HD-255 was lengthened to 1 and 3 h at 19°C, while little or no additional larvicidal effect was observed for exposure to different concentrations of Abbott powder 4606-125 (400–600 ITU/mg) longer than 60 min at 22°C (Frommer et al. 1980). As for chemical contact-insecticides, increases in exposure to *B.t.i.* paracrystalline bodies induces an increase in the mortality of all species; however, the presence of an optimal exposure duration after which the gain in mortality sharply declines to no significant additional effect, demonstrates the importance of a negative feedback on feeding behavior when using a stomach insecticide. Observations made by Lavergne[3] (personal communication 1983) on *S. decorum* exposed 20 min to 0.2 ppm of Teknar® (Zoecon Ltd.) at 12°C, showed a steady decline in adduction frequency of the labral fans only 30 min after inoculation, decreasing from 55 to 0 adductions/min/fan during the subsequent 60 min (water speed of 10 cm/s). Lacey and Federici (1979) also observed a decline in feeding rate of *S. vittatum* after only 30 min of exposure to 10 ppm of *B.t.i.* (HD-225) at 19°C; in less than one hour the labral fans when held only partially open. Because ingestion of the *B.t.i.* paracrystalline bodies is prerequisite to any toxic effect, this negative feedback on the feeding behavior limits the effective exposure period; while the minimum duration is dependent on the time required to ingest the minimum amount of

[3] Lavergne, A. 1983. Résultats préliminaires sur la filtration des larves de mouches noires (Diptera: Simuliidae), utilisant la cinématographie comme moyen d'observation. Unpublished report. Université du Québec à Trois-Rivières, Trois-Rivières, Québec, Canada G9A 5H7.

protoxin sufficient to induce irreversible damage to the larval gut epithelium.

The amounts of *B.t.i.* required to induce 50 and 90% of total mortality ($LC_{50\%}$and $LC_{90\%}$) on *Simulium decorum* and *Prosimulium mixtum/fuscum* larvae following a 30 min exposure at 4, 8, 12 and 18°C are shown in Table 1. These values exceed those calculated by Molloy et al. (1984) under similar conditions; they calculated at $LC_{50\%}$ of 0.14 ppm for a 15 min exposure on *Simulium vittatum* larvae (5th–6th instar) at 13°C. However, comparison with other experiments done with different formulations is difficult since, as stressed by Molloy et al. (1984), the potency expressed as I.T.U. mg^{-1} for *Aedes aegypti* (Linn.) has little correlation with the efficacy against black fly larvae, but is more closely related to the size of insecticide particles. Nevertheless, all laboratory trials of *B.t.i.* on black flies show that the amount of *B.t.i.* required to produce significant mortality, increases as exposure duration and temperature decrease (Gaugler and Finney 1982).

As summarized in figures 1 and 2, increase in exposure, concentration, and temperature essentially increases the mortality observed shortly after the beginning of the experiment, but produces little or no significant alteration in its subsequent progression; i.e., the major effect of an increase of these parameters is mainly achieved shortly after inoculation. Furthermore, increases in concentration and temperature not only increase the mortality of both species, but also shortens the onset of death (Fig. 2). An increase in concentration from 4 to 40 ppm shortens the onset of death by almost 24 h for *S. decorum* larvae at 4 and 8°C, and *P. mixtum/fuscum* larvae at 4°C. The same effect was observed when the water temperature was increased from 8 to 12°C on *S. decorum* larvae exposed 30 min to 4.0 ppm of *B.t.i.*. These data support the results obtained by Lacey and Federici (1979), who followed the progression of mortality caused by *B.t.* HD-261 in *S. vittatum* larvae over the temperature range 10–24°C; they found that for each increase of 4–5°C above 15°C not only did the percent mortality increase, but the onset of death was shortened by 24 h. Therefore, by considering the amount of δ-endotoxin being steadily activated at uniform temperature, pH and enzymatic activity (therefore proportional to the amount of ingested paracrystalline bodies), an increase in concentration, exposure, or temperature would proportionally increase the amount of toxic effects on midgut epithelial cells induced per unit of time; thus, as reflected by the sharp increase in mortality observed shortly after inoculation (2–4 h), enabling a physiological threshold to be reached more rapidly.

The rate at which temperature influences the relationship between duration of exposure, concentration, and total mortality is different for *Simulium decorum* and *Prosimulium mixtum/fuscum* larvae, as summarized in Fig. 3. For both species, the overall exposure duration and quantity of *B.t.i.* required to induce mortality decreases as temperature increases. However, the largest increase in larval mortality is observed when the temperature is raised from 4 to 8°C for *P. mixtum/fuscum* group, and from 8 to 18°C for *S. decorum* complex. This pattern is also illustrated by the lesser amount of *B.t.i.* needed to induce 50 and 90% total mortality ($LC_{50\%}$ and $LC_{90\%}$) at higher temperatures (Table 1). Significant proportional decreases of 7×, 145×, and 750× are observed in the $LC_{50\%}$ of *S. decorum* when the temperature is raised from 4 to 8°, 8 to 12°, and 12 to 18°C respectively. Likewise, proportional decreases of 17× and 3× are observed for *P. mixtum/fuscum* when the temperature is raised from 4 to 8° and 8 to 12°C. Other workers have also observed that total mortality of *S. vittatum* larvae decreases sharply at lower temperatures (Lacey and Federici 1979, Molloy et al. 1981). However, only Lacey and Federici (1979) mentioned that the decrease in mortality is more sensitive at temperatures between 15 and 10°C. These findings differ somewhat from those reported by

Table 1. Lethal concentrations (LC50% and LC95%) in ppm, of *Bacillus thuringiensis* var. *israelensis* (TEKNAR WDC) established for a 30 min exposure (calculated for 24 h postinoculation period).

Temp	*Simulium decorum*		*Prosimulium mixtum/fuscum*	
	LC50% (95% confidence limit)	**LC90%**	**LC50%** (95% confidence limit)	**LC90%**
4°C	5,000 (2,400–16,000)	1,100,000 (180,000–21,000,000)	170 (70.6–273)	1,800 (990–7,900)
8°C	660 (370–1,600)	42,000 (10,000–650,000)	10 (4–19)	440 (200–1,400)
12°C	4.5 (2.7–6.9)	220 (140–390)	3 (1.8–5.9)	140 (65–420)
18°C	0.006 (0.0001–0.04)	5.4 (1.6–29.9)		

Undeen and Nagel (1978) and Undeen and Berl (1979) who found little difference in the susceptibility of different species to *B.t.i.*. Nevertheless, it is clear that water temperature strongly affects the feeding behavior of black fly larvae, by increasing the frequency with which the labral fans and mandibles adduct in patterns particular to the species (Elsen and Hebrard 1979, Biggs 1985[4]; Lacoursière, unpublished data) and by concomittantly influencing growth and rate of survival (Ross and Merritt 1978). Lacey et al. (1978) observed that the feeding rates of *S. vittatum* larvae at 15, 19 and 24°C was significantly greater than those observed at lower temperatures (4 and 10°C), indicating a major temperature influence between 10 and 15°C for that species. Therefore, the differences in pattern of influence of the temperature on *B.t.i.* efficacy suggest a strong correlation to thermal adaptations. Immatures of *S. decorum* complex like those of *S. vittatum* are collected in streams of temperatures from 4 to 31°C, but are almost confined to warmer water; while larvae of the *Prosimulium mixtum/fuscum* group are commonly observed overwintering under ice cover, and are mostly collected in streams of temperatures from 0 to 14°C. Therefore, it seems that lower *B.t.i.* efficacy and difference in species response at low temperature is the result of mainly behavioral and physiological parameters of the larva.

Since the amount of particles ingested by a larvae is related to availability and feeding activity (Elsen and Hebrard 1979, Biggs 1985[4], Hart and Latta 1986), the efficacy of an insecticide requiring ingestion is not only dependent on the probability of encounter between toxic particles and insect, but is also dependent on the feeding behavior of the larva, as dictated by prevailing environmental conditions. Therefore, the notion of species "susceptibility" based on comparison of lethal concentrations determined at the same temperature does not reflect the "absolute" susceptibility of these species to that formulation since it can not resolve between the influence of the feeding activity or the formulation potency, at that precise temperature. Consequently the susceptibility of different species to an insecticide formulation requiring ingestion should be established in relation to an optimal level of activity, reflecting the range of preferred environmental conditions. This way, comparisons between species using "absolute susceptibility" would carry more biological information, while "effectiveness" of an insecticide (based on comparison of $LC_{\%}$ determined at the same temperature) bears only operational-control considerations. Consequently, by using "absolute susceptibility" we observe that *S. decorum* complex is "more susceptible" to *B.t.i.* protoxin than *P. mixtum/fuscum* group larvae (since the $LC_{50\%}$ of *S. decorum* larvae determined at 12 and 18°C are less then those of *P. mixtum/fuscum* group at 4 and 8°C), while the *B.t.i.* formulation is more effective on *P. mixtum/fuscum* group at low temperature.

Water alkalinization (pH 9) results in an increase in the mortality of *Simulium decorum* larvae exposed 30 min to 400 ppm of *B.t.i.*, whereas acidification (pH 4.5) results in a reduction in efficacy (Fig. 4). At 4°C, total mortality is increased by 58.3% (36.4 to 57.6%, $P = 0.015$) when pH is increased from neutral to pH 9, and decreased by 46.8% (36.4 to 19.4%, $P = 0.006$) when reduced to pH 4.5. No significant difference in the total mortality is apparent at 12°C as mortality is nearly 100% in all cases. However, the mortality observed 2 h after the beginning of the experiment is significantly increased by 51.2% (39.9 to 60.4%, $P = 0.022$) when pH is raised to 9, and is decreased by 51.6% (39.9 to 19.3%, $P = 0.014$) when pH is reduced to 4.5. These data support the results

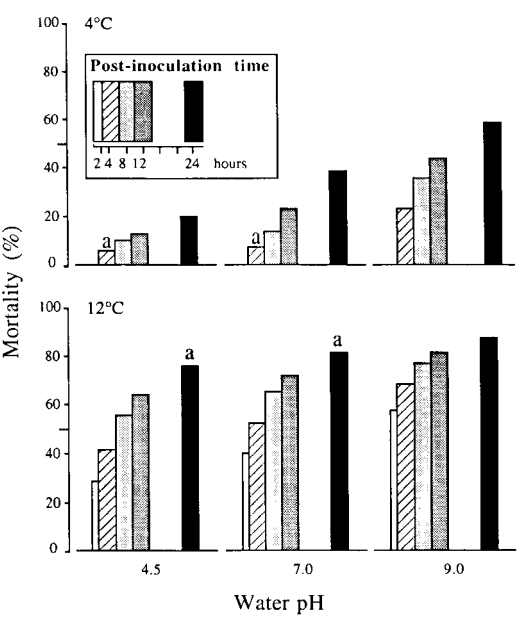

Fig. 4. Effect of water pH on *Bacillus thuringiensis* var. *israelensis* (TEKNAR WDC) efficacy on larvae of *Simulium decorum*, exposed 30 min to 400 ppm (Total mortality means crowned with the same letter are not significantly different at 5% error using a Kruskal-Wallis test).

[4] Biggs, J. 1985. A study of the filter-feeding behaviour of *Simulium* larvae (Diptera: Simuliidae). Ph.D. dissertation, Dept. of Zoology, Royal Holloway and Bedford New College, England.

of Lacey et al. (1978) who reported a positive correlation between increasing pH and pathogenicity of *B. thuringiensis* (HD-261) against *S. vittatum* larvae; although they did not demonstrate significant differences among the total mortality induced in each experiment. Investigations of product stability have shown that storage of aqueous *B.t.i.* formulations at pH 5.9 produces no variation in larvicidal activity, while storage at pH 9.0 results in loss of potency (Sinègre et al. 1980). Alkaline condition of the insect gut is necessary to dissolve the paracrystalline bodies, allowing further endotoxin activation by the proteolytic enzymes associated with protein digestion (Lacey et al. 1978). Consequently, exposure of the paracrystalline bodies to an alkaline environment could "destabilize" the paracrystalline structure making it more accessible for further break down and activation after ingestion by the larvae. Further, acidic conditions could "over-stabilize" the paracrystalline structure making it less susceptible to rapid dissolution and activation. These results differ somewhat from those reported by Sinègre et al. (1980) and Ignoffo et al. (1981) who suggested that pH values characteristic of black fly habitats are not likely to affect *B.t.i.* activity. However, it is not unusual to encounter streams with a natural acidity of pH 4.5 and 5 in the Canadian Shield, while pHs of 8 to 9 are not uncommon in western Canada. Since no data on the influence of water pH on physiological conditions and feeding behavior of black fly larvae are available, it is impossible at this point to resolve between the possible effect of the pH on the larval feeding and the formulation potency.

Following alkaline dialysis of the *B.t.i.* preparation for 2 h at pH 11, we observed an increase of 62.2% (39.9 to 64.7%, $P = 0.01$) in the mortality observed after 2 h, and 16.9% (80.3 to 93.9%, $P = 0.015$) in total mortality of *S. decorum* larvae at 12°C (30 min exposure to a 400 ppm inoculum). This suggests that extralarval alkaline hydrolysis can affect the *B.t.i.* efficacy. This alkaline prehydrolysis of the paracrystalline bodies could therefore be used in cold and acidic environments to compensate for loss of efficacy. However, the effect of pH on *B.t.i.* efficacy deserves closer scrutiny as nothing is known about its influence on larval behavior and physiology, nor on its effect on *B.t.i.* selectivity.

CONCLUSION

In the search for safe, economical black fly larvicide, the entomopathogen *Bacillus thuringiensis* var. *israelensis* has emerged as the most likely alternative to chemical larvicides. However, as noted by Gaugler and Finney (1982), many of the parameters influencing *B.t.i.* efficacy have been determined using bioassays on mosquitoe larvae, and should be reexamined with black fly larvae because of behavioral, physiological and habitat differences. Recent studies have shown that hydrodynamic parameters play a major role in shaping the feeding behavior of lotic organisms. Better understanding of the filtration process could lead to a more efficient use of existing tools in black fly pest management.

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