Biocontrol Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/cbst20

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Accepted author version posted online: 21 Jan 2014.Published online: 28 Apr 2014.

To cite this article: Yanina Mariottini & Carlos Ernesto Lange (2014) Infectivity, viability and effects of Paranosema locustae (Microsporidia) on juveniles of Dichroplus maculipennis (Orthoptera: Acrididae: Melanoplinae) under laboratory conditions, Biocontrol Science and Technology, 24:6, 715-722, DOI: 10.1080/09583157.2014.884994

To link to this article: http://dx.doi.org/10.1080/09583157.2014.884994

Biocontrol Science and Technology, 2014
Vol. 24, No. 6, 715–722, http://dx.doi.org/10.1080/09583157.2014.884994

SHORT COMMUNICATION

Infectivity, viability and effects of *Paranosema locustae* (Microsporidia) on juveniles of *Dichroplus maculipennis* (Orthoptera: Acrididae: Melanoplinae) under laboratory conditions

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(*Received 30 October 2013; returned 2 December 2013; accepted 15 January 2014*)

Infectivity and effects on host of a long-term stored aqueous suspension of *Paranosema locustae* on juveniles of *Dichroplus maculipennis,* a pest grasshopper in parts of the Pampas and Patagonia, were evaluated. Infections developed in 90–97.8% of treated individuals. Mortality increased with time, reaching highest values at 30–40 days post-inoculation (79.5–100%). Infected nymphs showed significantly slower development.

Keywords: biocontrol agent; *Dichroplus maculipennis*; grasshopper; mortality; spore viability; *Paranosema locustae*

Dichroplus maculipennis (Blanchard) is one of the most widely distributed species of the genus, occurring in southernmost Brazil (Río Grande do Sul), much of Argentina and Chile, and Uruguay (COPR, 1982). The microsporidium *Paranosema locustae* (Canning), developed in the USA as a long-term biocontrol agent of grasshoppers (Solter, Becnel, & Oi, 2012), was introduced in Argentina in 1978–1982 and 1996 and became established in grasshopper populations in the western Pampas and two areas in north-western Patagonia (Bardi, Mariottini, Plischuk, & Lange, 2012; Lange & Azzaro, 2008; Lange & Cigliano, 2005). Infections have been detected in 22 grasshopper species, mostly melanoplines (subfamily Melanoplinae), including *D. maculipennis*. However, infected *D. maculipennis* were recorded in the Patagonian sites only, not in Pampas, despite extensive monitoring through many years (Bardi et al., 2012). Therefore, we evaluated the infectivity, mortality and effects on host development of *P. locustae* on juveniles of *D. maculipennis* from the Pampas in the laboratory. In addition, due to the source of the inoculum we employed, the study provided further information on the laboratory viability of *P. locustae* spores after extreme long-term storage as frozen aqueous suspensions.

Following the lettuce leaf disc procedure (Habtewold, Landin, Wennergen, & Bergman, 1995; Hildreth, Brey, Fuller, & Foster, 2000), we orally inoculated known amounts of *P. locustae* spores to third-instar nymphs (two days after moulting) of *D. maculipennis* from the rearing facilities of the Centre for Parasitological Studies and Vectors (CEPAVE), established from individuals collected in Laprida county

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(36°02′S–59°06′W) in the southern Pampas (Buenos Aires province), an area where infections by *P. locustae* have never been found in spite of intensive surveys (Bardi et al., 2012). Forty-six nymphs were individually treated with 10^5 spores each, 40 nymphs with 10^4 spores each and 50 nymphs were fed with a disc each having no spores (controls). After inoculation, nymphs were individually kept in cylindrical sheet acetate tubes with screened ends (16.5 cm long × 4.7 cm diameter; Henry, 1985) under controlled conditions (30°C; 14:10, L:D photoperiod; 40% relative humidity), fed on thoroughly washed pieces of corn and lettuce leaves and wheat bran, and checked twice a day for mortality and instar of development until death or until day 40, when the experiment was terminated. Disease diagnosis was done microscopically (400×, 1000×) after either dissection or homogenisation of grasshoppers as previously described (Lange & Cigliano, 2010; Lange, Sánchez, & Wittenstein, 2000). Given that spores of *P. locustae* in aqueous suspensions are known to remain viable even after extreme prolonged frozen storage (Lange, 1997), we utilised remnants of the spore concentrate employed in the introductions made in the Pampas in the early 1980s. The concentrate was produced by Bio-Ecologists, Inc. (Denver, CO, USA; Lange & de Wysiecki, 1996) and remnants were stored at −14°C to −32°C since then. Dilutions of the spore stock to desired concentrations were made in double distilled water by means of a haemocytometer as described by Undeen & Vávra (1997).

The proportion of infected individuals in each of the two treatments and controls was compared with a proportion test (Xlstat-Pro 7.5.3, 2005). A repeated measures analysis of variance (ANOVA) was used to compare mortality differences between controls and treatments at 10, 20, 30 and 40 days post-inoculation (dpi). Mortality values of treatments were corrected with Abbott's formula (Abbott, 1925) and were arcsine root transformed (Quinn & Keough, 2002). Before the analysis, the Mauchly sphericity test (Scheiner & Gurevitch, 2001) was performed. One-way ANOVA to compare the differences in duration of each nymphal stage between control and treatments was conducted. In both analyses, a Tukey's honestly significant difference (HSD) was used for later comparison. Statistica 7.0 (Stat Soft Inc, 2004) was used to calculate the ANOVA.

While there were no infections in controls, infections developed in 97.8% of individuals treated with 10^5 spores and in 90% of those treated with 10^4 spores, a non-significant difference (proportion test, $Z_0 = 1.304$, $p = 0.192$). Whilst 80% of control individuals reached adulthood, only 19.6% made it at the higher dose, all were infected and all died before the end of the assay. With the lower dose, 17.5% of individuals reached adulthood, and four of them were uninfected and still alive at termination. The difference in percentage of insects reaching adulthood at higher and lower doses was not significant (proportion test, $Z_0 = 0.246$, $p = 0.806$). Infected adults showed morphological abnormalities such as twisted wings and legs. They also exhibited lethargic behaviour and appeared to consume less food.

The mortality analysis showed significant differences for both factors (dose, dpi) and for their interaction (Table 1). While control mortality was constant during the 40 days of the assay, the mortality registered in treatments increased significantly with time. Mortality at 20, 30 and 40 dpi was significantly higher in treatments than in controls (Tukey test $p < 0.05$) and also higher than in previous intervals (Table 2). The values for days 21–30 and 31–40 (100 ± 0 for 10^5, 79.51 ± 20.48 for 10^4) were significantly higher than all other values observed in this study (Tukey test $p < 0.05$).

Table 1. Results of repeated measures ANOVA analysis of mortality on *Dichroplus maculipennis* by *Paranosema locustae* for treatments (dose) factor, time period factor and the interaction between them (Mauchly sphericity test: χ^2: 7.38, DF: 5 p: 0.19).

	DF effect	DF error	F value	p
Treatments	2	9	43.6	<0.0001
Time periods	3	27	20.8	<0.0001
Treatments × time periods	6	27	6.1	0.00038

Nymphal development of *D. maculipennis* was significantly longer in treated fourth (ANOVA: DF = 2, $F = 5.18$, $p = 0.007$), fifth (ANOVA: DF = 2, $F = 12.04$ $p \leq 0.0001$) and sixth (ANOVA: DF = 2, $F = 2.29$, $p \leq 0.0001$) instars (Table 3). Duration of the sixth instar at both higher and lower doses was twice of that in controls. Differences in the duration of nymphal instars of individuals treated with different doses of *P. locustae* were not significant (Table 3).

Similar experimental inoculations with *P. locustae* to the ones we performed have been conducted against a number of different grasshopper species such as the melanoplines *Baeacris punctulatus*, *Dichroplus elongatus*, *Dichroplus pratensis*, *Melanoplus bivittatus*, *Melanoplus sanguinipes* and *Melanoplus differentialis*, the Cyrtacanthacridinae *Schistocerca gregaria* and *Schistocerca cancellata*, the

Table 2. Percent mortality occurring in each 10-day interval for *Dichroplus maculipennis* either left untreated or treated with *Paranosema locustae* at 10^5 or 10^4 spores per individual.

	Mortality at intervals after treatment			
	Days 1–10	Days 11–20	Days 21–30	Days 31–40
Control	5.9 ± 3.4 Aa	2.1 ± 2.1 Aa	4.3 ± 2.1 Aa	2.4 ± 2.4 Aa
10^5	6.6 ± 4.6* Aa	9.4 ± 6.7 Aa	51.2 ± 12.9 Bb	100 ± 0 Bc
10^4	8.6 ± 3.8 Aa	10.8 ± 4.8 Aa	44.4 ± 15.5 Bb	79.5 ± 20.5 Bc

Note: Different upper case letters in the same columns and different lower case letter in the same lines indicate significant differences ($p < 0.05$; Tukey test).
*The percent mortalities for treatments have been corrected for control mortality using Abbott's (1925) formula and were arcsine root transformed for statistical analysis.

Table 3. Duration in days (mean ± ES) of each nymphal instars of *Dichroplus maculipennis* and maximum and minimum values in parenthesis (brackets) at 30°C, 14:10 L:D.

	Instars			
	Third	Fourth	Fifth	Sixth
Control	5.9 ± 0.1a (4–8)	6.3 ± 0.3b (3–10)	6.8 ± 0.2b (3–12)	6.1 ± 0.2b (3–11)
10^5	5.5 ± 0.1a (3–7)	7.5 ± 0.5a (4–18)	9.6 ± 0.6a (3–16)	13.2 ± 0.7a (7–21)
10^4	5.7 ± 0.2a (4–9)	7.9 ± 0.8a (4–31)	10.2 ± 0.8a (4–19)	14.1 ± 1.0a (7–20)

Note: Different letters in a column indicate significant differences ($p < 0.05$; Tukey test).

Table 4. Infectivity percentage, mortality percentage at different days post-inoculation (dpi) and recording of delayed development in juveniles of various grasshopper and locust species of different subfamilies following experimental inoculation of *Paranosema locustae* under laboratory conditions.

Grasshopper subfamily/species	Dose	Instar	% Infectivity	% Mortality (dpi) 19–20	25	30	40	Delayed development	Reference
Melanoplinae									
Melanoplus bivittatus	5.5×10^5	III	–	–	50	–	–	yes	Henry (1978)
Melanoplus differentialis	$2.0 \times 10^{3–5}$	IV	80.8–96.2	–	–	–	–	yes	Henry and Oma (1981)
Melanoplus sanguinipes	1.0×10^5	III	–	50	–	–	–	yes	Henry and Oma (1981), Henry (1990), Hildreth et al. (2000)
Baeacris punctulatus	1.0×10^5	III	83.3	–	–	70	–	–	Lange (1997).
	2.4×10^4	III	73.3			50			
Dichroplus elongatus	2.4×10^4	III	93.3	–	–	60	–		
Dichroplus pratensis	2.4×10^4	III	100	–	–	97	–		
Dichroplus maculipennis	1.0×10^4	III	90	10.8	–	44.4	79.5 100	yes	Present study
	1.0×10^5	III	97.8	9.4		51.2			
Cyrtacanthacridinae									
Schistocerca cancellata	1.0×10^5	III	52.5	17.5	–	35	–	yes	Lange et al. (2000)
Schistocerca gregaria	3.2×10^4	III–IV		–	–	44.7–55.8	–	yes	Tounou, Kooyman, Douro-Kpindou, Gumedzoe, and Poehlingn (2011)
	5.6×10^6	III–IV	74.8–100			76.9–77.5			
	1.0×10^7	III–IV	–			93.7–94.6			
Oedipodinae									
Aiolopus longicornis	1.0×10^7	III	100	–	62?	–	–	–	Habtewold et al. (1995)
Oedaleous senegalensis	3.2×10^4	III	100	–	–	57.9	–	yes	Tounou et al. (2011)
	5.6×10^6	III	100			71.1			
	1.0×10^7	III	100			88.5			
Locusta migratoria migratorioides	1.5×10^6	IV	–	13.3–24.3	–	10.3–33.3	88.8–90.9	yes	Raina, Das, Rai, and Khurad (1995)

Table 4 (*Continued*)

Grasshopper subfamily/species	Dose	Instar	% Infectivity	% Mortality (dpi)				Delayed development	Reference
				19–20	25	30	40		
Locusta migratoria manilensis	5.0×10^6	III	99.6	50	–	–	–		Zhou and Zhang (2009)
Gomphocerinae									
Rhammatocerus schistocercoides	1.0×10^6	III	40	15	–	–	–	–	Silva, Magalhaes, and Teixeira (1996)
Romaleinae									
Tropidacris collaris	1.0×10^5	III	38	–	–	–	–	–	Lange, Bardi, and Plischuk (2008)

Oedipodinae *Aiolopus longicornis*, *Oedaleous senegalensis* and *Locusta migratoria*, the Gomphocerinae *Rhammatocerus schistocercoides* and the Romaleinae *Tropidacris collaris* (Table 4). Species in the subfamilies Oedipodinae and Melanoplinae, followed by the Gomphocerinae are usually more susceptible than species in other subfamilies (Lange, 2005). A comparison with those studies indicates that in the laboratory and in terms of infectivity, mortality and effects on host, the kind of response of *D. maculipennis* from the Pampas to inoculation with *P. locustae* followed the trend expected for a clearly susceptible host. Infectivity and mortality were high, and host development was typically altered. In fact, although some differences exist, such as the time to high mortality, the response was quite close to the responses known for three North American species of *Melanoplus* (*M. bivittatus*, *M. differentialis*, *M. sanguinipes*) which are natural hosts (as defined by Onstad et al., 2006) of *P. locustae*. More rigorous, accurate comparisons cannot be done because different conditions and doses were employed. As it stands today, we found intriguing the lack of detection of *P. locustae* in *D. maculipennis* of the Pampas, especially considering that the searches conducted were able to detect the pathogen in 21 other grasshopper species (Bardi et al., 2012), and infections are common in *D. maculipennis* in establishment areas of north-western Patagonia (Lange & Azzaro, 2008; Lange & Cigliano, 2010). Therefore, there is currently no clear explanation for the apparent lack of infection of *D. maculipennis* in the Pampas.

Another noteworthy outcome of our inoculations is the extremely prolonged time that *P. locustae* spores remained viable as frozen aqueous suspensions when tested in the laboratory. Although the ability of *P. locustae* to withstand long-term, low temperature storage was noted previously (13 years; Lange, 1997), we have found good survival even after ~30 years. Since Henry & Oma (1974) found that stored spores were less effective in field essays than 'fresh spores' (obtained within 4 months before use), the long-term viability we have observed should be checked under field conditions.

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