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

Taylor & Francis
Taylor & Francis Group

Downloaded by [Carlos E. Lange] at 11:51 30 April 2014

SHORT COMMUNICATION

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

Yanina Mariottini[a]* and Carlos Ernesto Lange[a,b]

[a]*Centro de Estudios Parasitológicos y de Vectores (CEPAVE), CCT La Plata CONICET, Universidad Nacional de La Plata (UNLP), La Plata, Argentina;* [b]*Comisión de Investigaciones Científicas (CIC) de la Provincia de Buenos Aires, La Plata, Argentina*

(*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

*Corresponding author. Email: ymariottini@cepave.edu.ar

© 2014 Taylor & Francis

(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 $p < 0.05$).

Downloaded by [Carlos E. Lange] at 11:51 30 April 2014

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).

Downloaded by [Carlos E. Lange] at 11:51 30 April 2014

Downloaded by [Carlos E. Lange] at 11:51 30 April 2014

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)

Downloaded by [Carlos E. Lange] at 11:51 30 April 2014

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.

Downloaded by [Carlos E. Lange] at 11:51 30 April 2014

References

Abbott, W. S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology*, *18*, 265–267.

Bardi, C., Mariottini, Y., Plischuk, S., & Lange, C. E. (2012). Status of the alien pathogen *Paranosema locustae* (Microsporidia) in grasshoppers (Orthoptera: Acridoidea) of the Argentine Pampas. *Biocontrol Science and Technology*, *22*, 497–512. doi:10.1080/09583157.2012.665023.

COPR. (1982). *The locust and grasshopper agricultural manual*. London: Centre for Overseas Pest Research, 690 p.

Habtewold, T., Landin, J., Wennergen, U., & Bergman K. O. (1995). Life table for the tef grasshoppers, *Aiolopus longicornis*, under laboratory conditions and demographic effects of the pathogen *Nosema locustae*. *Biological Control*, *5*, 497–502. doi:10.1006/bcon.1995.1059

Henry, J. E. (1978). Microbial control of grasshoppers with *Nosema locustae* Canning. *Miscellaneous Publications of the Entomological Society of America*, *11*, 85–95.

Henry, J. E. (1985). *Melanoplus* spp. In P. Singh & R. F. Moore (Eds.), *Handbook of insect rearing* (vol. 1, pp. 451–464). Amsterdam: Elsevier.

Henry, J. E. (1990). Control of insects by Protozoa. In R. Baker & P. E. Dunn (Eds.), *New directions in biological control: Alternatives for suppressing agricultural pests and diseases* (pp.161–176). New York, NY: Alan Liss.

Henry, J. E., & Oma, E. A. (1974). Effect of prolonged storage of spores on field applications of Nosema locustae (Microsporida: Nosematidae) against grasshoppers. *Journal of Invertebrate Pathology*, *23*, 371–377. doi:10.1016/0022-2011(74)90104-9

Henry, J. E., & Oma, E. A. (1981). Pest control by *Nosema locustae*, a pathogen of grasshoppers and crickets. In D. Burges (Ed.), *Microbial control of pests and plant diseases 1970–1980* (pp. 573–586). New York, NY: Academic Press.

Hildreth, M. B., Brey, Ch. W., Fuller, B. W., & Foster, R. N. (2000). Laboratory bioassays of *Nosema locustae*. In G. L. Cinningham & M. W. Sampson, *Grasshopper integrated pest management. User handbook* (pp. I.3: 1–7). Washington: USDA-APHIS Tech. Bull., # 1809.

Lange, C. E. (1997). Viabilidad del acridicida *Nosema locustae* (Protozoa: Microspora) luego de almacenamiento prolongado. *Revista de la Sociedad Entomológica Argentina*, *56*, 63–65.

Lange, C. E. (2005). The host and geographical range of the grasshopper pathogen *Paranosema (Nosema) locustae* revisited. *Journal of Orthoptera Research*, *14*, 137–141. doi:10.1665/1082-6467(2005)14[137:THAGRO]2.0.CO;2

Lange, C. E., & Azzaro, F. G. (2008). New case of long-term persistence of *Paranosema locustae* (Microsporidia) in melanopline grasshoppers (Orthoptera: Acrididae: Melanoplinae) of Argentina. *Journal of Invertebrate Pathology*, *99*, 357–359. doi:10.1016/j.jip.2008.09.002

Lange, C. E., Bardi, C., & Plischuk, S. (2008). Infectividad del agente de biocontrol *Paranosema locustae* (Microsporidia) para la "tucura quebrachera", *Tropidacris collaris* (Orthoptera: Romaleidae), en condiciones de laboratorio. *Revista de la Sociedad Entomológica Argentina*, *67*, 151–155.

Lange, C. E., & Cigliano, M. M. (2005). Overview and perspectives on the introduction and establishment of the grasshopper biocontrol agent *Paranosema locustae* (Microsporidia) in the western Pampas of Argentina. *Vedalia*, *12*, 61–84.

Lange, C. E., & Cigliano, M. M. (2010). Prevalence and infection intensity of the biocontrol agent *Paranosema locustae* (Microsporidia) in field-collected, newly-associated hosts (Orthoptera: Acrididae: Melanoplinae). *Biocontrol Science and Technology*, *20*, 19–24. doi:10.1080/09583150903338746

Lange, C. E., & De Wysiecki, M. L. (1996). The fate of *Nosema locustae* (Microsporida: Nosematidae) in argentine grasshoppers (Orthoptera: Acrididae). *Biological Control*, *7*, 24–29. doi:10.1006/bcon.1996.0059

Lange, C. E., Sánchez, N. E., & Wittenstein, E. (2000). Effects of the pathogen *Nosema locustae* (Protozoa: Microspora) on mortality and development of nymphs of the South American locust, *Schistocerca cancellata* (Orthoptera: Acrididae). *Journal of Orthoptera Research, 9*, 77–80. doi:10.2307/3503637

Onstad, D. W., Fuxa, J. R., Humber, R. A., Oestergaard, J., Shapiro-Ilan, D. I., Gouli, V. V., … Lacey, L. A. (2006). *An abridged glossary of terms used in invertebrate pathology*. (3rd ed.). Knoxville, TN: SIP. Retrieved from http://sipweb.org/glossary

Quinn, G., & Keough, M. (2002). *Experimental design and data analysis for biologist* (p. 509). Cambridge: Cambridge University Press.

Raina, S. K., Das, S., Rai, M. M., & Khurad, A. M. (1995). Transovarial transmission of *Nosema locustae* (Microspora: Nosematidae) in the migratory locust *Locusta migratoria migratorioides*. *Parasitology Research*, *81*, 38–44. doi:10.1007/BF00932415

Scheiner, S. M., & Gurevitch, J. (2001). *Design and analysis of ecological experiments* (2nd ed.). New York, NY: Oxford University.

Silva, J. B. T., Magalhaes, B. P., & Teixeira, A. B. (1996). Pathogenecity of *Nosema locustae* (Protozoa: Microspora) against *Rhammatocerus schistocercoides* Rehn (O:A) and *Stiphra robusta* Mello-Leitao (Orthoptera: Proscopiidae). *Annais Sociedade Entomologica do Brasil*, *25*, 545–547.

Solter, L. F., Becnel, J. J., & Oi, D. H. (2012). Microsporidian entomopathogens. In F. E. Vega & H. K. Kaya (Eds.), *Insect pathology* (2nd ed.; pp. 221–263). London: Elsevier.

Stat Soft Inc. (2004). *STATISTICA (data analysis software system), version 7*. Retrieved from www.statsoft.com

Tounou, A. K., Kooyman, C., Douro-Kpindou, O., Gumedzoe, Y. M., & Poehlingn, H. M. (2011). Laboratory assessment of the potential of *Paranosema locustae* to control immature stages of *Schistocerca gregaria* and *Oedaleus senegalensis* and vertical transmission of the pathogen in host populations. *Biocontrol Science and Technology*, *21*, 605–617. doi:10.1080/09583157.2011.566323

Downloaded by [Carlos E. Lange] at 11:51 30 April 2014

Undeen, A. H., & Vávra, J. (1997). Research methods for entomopathogenic Protozoa. In Lacey, L. (Ed.), *Manual of techniques in insect pathology* (pp. 117–151). San Diego, CA: Academic Press.

XLSTAT-PRO. (2005). *User's guide, version 7.5.3*. New York, NY: Addinsoft Inst Inc. Retrieved from http://www.xlstat.com/en/home

Zhou, X., & Zhang, L. (2009). Selection of *Antonospora locustae* (Protozoa: Microsporidae) with higher virulence against *Locusta migratoria manilensis* (Orthoptera: Acrididae). *Biocontrol Science and Technology*, *19*, 421–427. doi:10.1080/095831509027925

Downloaded by [Carlos E. Lange] at 11:51 30 April 2014