Development of a peroral, droplet-dose bioassay laboratory technique and its application on a granulovirus for *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae)

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The false codling moth, *Thaumatotibia (= Cryptophlebia) leucotreta* Meyrick (1912) (Lepidoptera: Tortricidae) is regarded as a serious pest on citrus and other crops in Africa south of the Sahara (Newton 1998). Biological pesticides have been formulated using entomopathogenic viruses such as granuloviruses (Baculoviridae) (Hunter-Fujita et al. 1998). The efficacy of registered biological control formulations using naturally-occurring South African *Cryptophlebia leucotreta* granulovirus (CreGV-SA) needs to be determined as close to field conditions as possible. This is necessary as *T. leucotreta* eggs are laid on the surface of the fruit and CreGV-SA biopesticides target the neonate larvae before they burrow into the fruit, where they remain until pupation (Newton 1998). Therefore there is only one chance for larvae to ingest virus (Lacey 1997). An understanding of these pathogen–host interactions and an ability to measure these properties is needed for the selection of effective isolates and formulations (Hughes et al. 1986). There is a need to quantitatively measure the potency of preparations accurately (Hughes et al. 1986). Due to its precision, the peroral droplet technique is widely used for determining the lethal dose of baculoviruses (Bouwer & Avyidi 2006; Kunimi & Fuxa 1996; Smits & Vlak 1988).

The aim of this study was to develop an appropriate and accurate droplet feeding assay to test the virulence of purified granulovirus against *T. leucotreta* and to propose an adapted dose-response technique that could be used in other studies involving lepidopteran pests with very small neonates.

Bioassay technique preparation. All larvae in the experiments were used within 24 hours of hatching and were fed on autoclaved artificial diet (Moore 2002) only after ingesting virus particles. The bioassay methods were modified from Lacey (1997) and were not carried out in bioassay trays as described in Moore et al. (2011). Instead, bioassays were conducted in independent, size 10 plastic tablet vials (supplied by Grahamstown Pharmacy) as the original bioassay trays were susceptible to severe fungal contamination (*Aspergillus* spp.) and subject to cross-contamination of the virus, as larvae were able to move freely between cells. Vial-tray sets of 25 individual tablet vials were fastened together and used for each treatment. These prevented horizontal transmission of virus, reduced fungal contamination considerably and prevented cannibalism amongst neonate larvae (Annecke & Moran 1982). The diet was sliced into plugs and placed into the vials in a laminar flow cabinet. This technique was used in all bioassay experiments.

Determining larval droplet ingestion. *Thaumatotibia leucotreta* neonate larvae are too small (1–2 mm in length) to manually feed a specified amount of virus accurately. The volume ingested was calculated using larval weights before and after ingestion. Parafilm was placed on the inside of the lids of jam jars (350 ml) which contained only the hatching eggs. Once the Parafilm® contained over 100 larvae it was removed and placed in a Petri dish. Ten larvae were selected using a size 000 paintbrush and weighed together using a Sartorius electronic micro-balance (Model: MC 5, Sartorius AG, Germany). From this, the average weight of an individual larva was estimated. These larvae were discarded and not used again to prevent excess stress caused by over handling that may in turn affect the volumes ingested. About 20 10-μl droplets of distilled water containing 1 % (w/v) brilliant blue dye (Navon & Ascher 2000) were placed randomly amongst the remaining larvae on the Parafilm®. Ingestion was confirmed by the presence of a fully blue-stained gut using a Leica EZ4D dissecting microscope (magnification ×10), and 10 larvae were selected and then weighed. The weighing of larvae was replicated 10 times using different larvae each time. Ten 50 μl dye-droplets were weighed separately. The weight of liquid ingested was then calculated and from this the volume ingested was determined.