Regular Paper

SYMBIOFAUNA ASSOCIATED WITH THE REPRODUCTIVE SYSTEM OF *Cotesia flavipes* AND *Doryctobracon areolatus* (HYMENOPTERA, BRACONIDAE)

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ABSTRACT

The diversity of symbionts associated with insects and the range of effects they exert on their hosts have prompted studies to understand the role these microorganisms may have on host biology, particularly in relation to the interaction of their hosts with other trophic levels. There is also a possibility of using such symbionts as vectors of genes for insect control or learning on the mechanisms they use to interact with their hosts for the development of new approaches to insect control. Since most of these symbionts are transmitted transovarially from one generation to another, we used electron microscopy to assess the occurrence and morphology of these microorganisms in the female reproductive tissues of two important insect pest parasitoids, the braconids *Cotesia flavipes* and *Doryctobracon areolatus. Cotesia flavipes* was associated with a polydnavirus (PDV), whereas *D. areolatus* harbored the rickettsia-like bacterium *Wolbachia*. The ultrastructural morphology and localization of these symbionts in their host ovaries are described. None of the populations of the species studied were associated with non-PDV particles, and their sole association with specific symbionts will facilitate studies on the role of these symbionts in the association with their hosts, and on the associations of their hosts with other trophic levels.

Key words: Bracovirus, biological control, fruit flies, host-parasitoid interactions, host regulation, sex-ratio distorter

INTRODUCTION

The widespread association of insects with microorganisms, the variety of processes involved in these associations and the role they may have on the interaction of their hosts with other trophic levels have amused researchers for decades [9,27]. Although it is often difficult to distinguish between weakly aggressive or beneficial and neutral associations, symbiosis has provided an evolutionary strategy for eukaryotes to gain access to a wider range of metabolic resources. Endosymbiosis is very common in insects and involves mainly bacteria, although certain groups of insects are also mutualistically associated with virus-like particles [16,18,38]. Since symbiosis plays an important role in insect population biology, this phenomenon has been investigated as a means of developing new strategies for insect pest control [4,6,14,38].

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Insect parasitoids are extensively used in biological control programs and have unique mutualistic associations with virus-particles, although many may also harbor symbiotic bacteria [16,22]. Parasitoids form a very diverse group that has undergone ecological and physiological specializations to locate and exploit their hosts in extremely varied ecological systems and at any developmental stage [40,46,48]. The biological success of parasitoids reflects a number of adaptations that have been developed through the evolutionary history of their association with host insects and plants [13,30].

Parasitoids have also developed a natural arsenal and a number of physiological mechanisms to enable them to successfully colonize the host and regulate host development to their own benefit [5,43,47]. The successful exploitation of the mechanisms that parasitoids use to control insect development represents a promising approach for the development of new molecules or strategies for insect pest control [6,37]. One of the most studied mechanisms insects use to subdue and regulate their host development is through a symbiotic association with polydnaviruses

(PDVs) [7], which have unusually large, segmented genomes composed of 10 to 25 circular DNA segments. PDVs are obligate symbionts of Braconidae and Ichneumonidae parasitoid wasps and are required for the successful parasitism and suppression of the host immune system, as well as for inducing physiological alterations in the parasitized host, including disruption of the development and mobilization of protein stores for parasitoid utilization [2,22,28,34,42]. Although PDV particles have been extensively studied, non-PDV viruses are also being investigated and may be in the process of co-evolving towards a symbiotic relationship with their wasp vectors, in a manner similar to PDVs [23].

Symbiotic bacteria have also been reported associated with a number of parasitoid species, with the sex regulators bacteria being the most frequent ones. Among the sex regulators bacteria found in insects, *Wolbachia* infect a large number of species, including parasitic hymenopterans [21,52]. These bacteria affect insect reproduction in different ways [39,51], and selected lines of *Wolbachia* have been investigated for their potential use to host control because of their ability to modulate the sex ratio or to act as vectors for introducing foreign genes into their hosts, which could open up new opportunities for insect manipulation [19,25,35]

The goal of this work was to study the occurrence and morphology of the symbionts associated with the female reproductive system of the braconids *Cotesia flavipes* (Cameron) and *Doryctobracon areolatus* (Szépligeti), which are parasitoids of lepidopteran moths and fruitflies [8,31], respectively. This information could be helpful in assessing the potential usefulness of these symbionts in insect pest control programs.

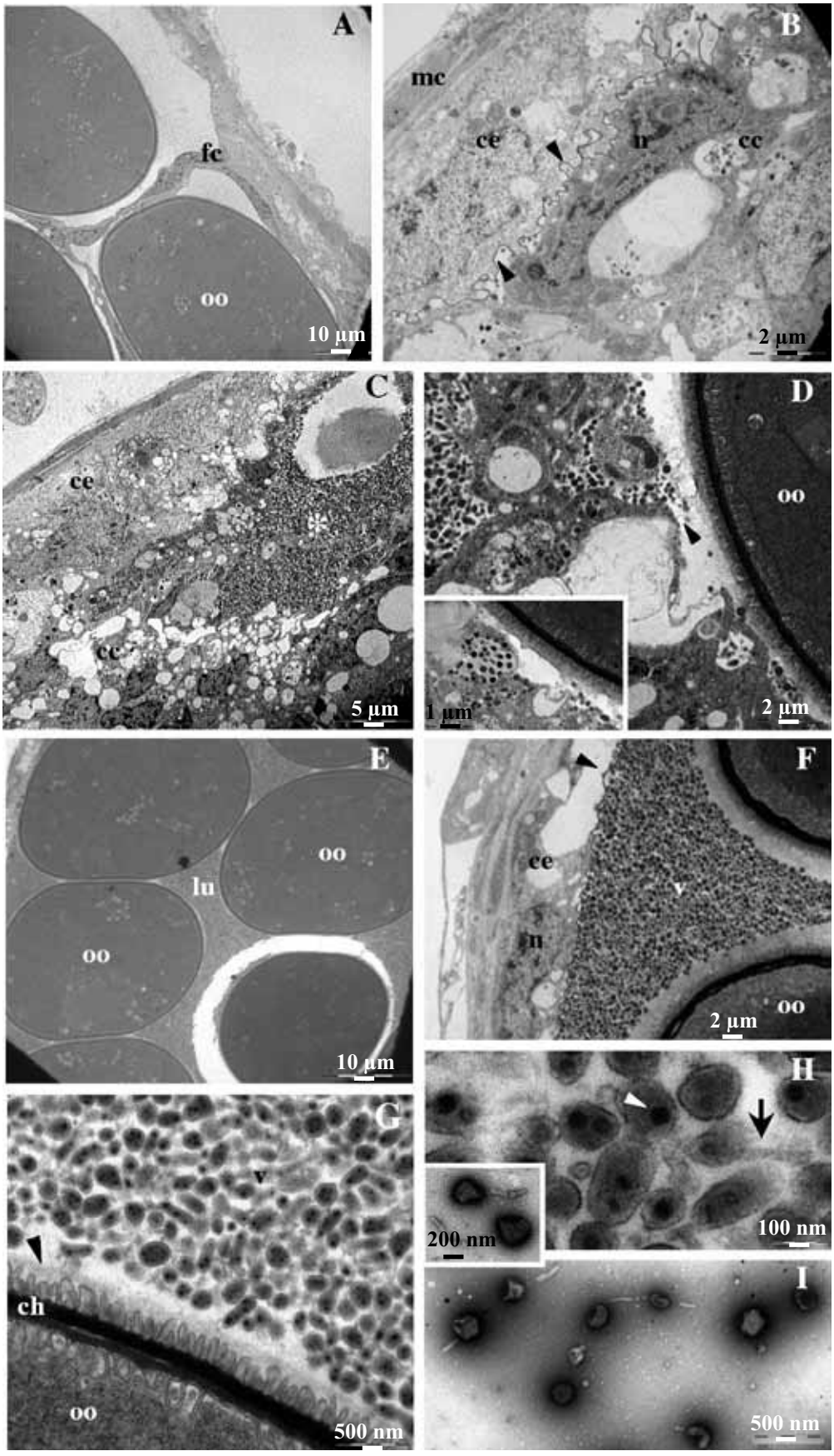
MATERIAL AND METHODS

Parasitoids. Cotesia flavipes (Cameron) females were obtained from a stock culture of a strain maintained in laboratory conditions on its natural host, the sugarcane borer *Diatraea saccharalis* (Fabricius), which is mass produced and released throughout the sugarcane-growing regions of Brazil. Females of *Doryctobracon areolatus* (Szépligeti) were obtained from parasitized fruitflies collected from infested fruits at the Campus of the Escola Superior de Agricultura Luiz de Queiroz (ESALQ/USP), in Piracicaba, São Paulo State.

Transmission electron microscopy [26]
Ultrastructural analysis: Fifteen to 20, 2-4-day-old parasitoid females were decapitated in 0.85% NaCl saline and the reproductive system was removed from the abdomen by gently pulling on it by the ovipositor while holding the female thorax with a pair of forceps. The ovary, accessory glands and venom reservoir were cleaned of the remaining adherent tissue, washed twice in clean saline and fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) to which 1.8% sucrose (R. Dallai, personal communication) was added. Samples were fixed at 4°C for 4 h, washed twice in cacodylate buffer and then fixed in 1% osmium tetroxide in cacodylate buffer for 1 h at 4°C followed by dehydration in a graded series of acetone prior to embedding in a low viscosity epoxy resin (Spurr). Ultrathin sections obtained with a Leica EM UC6 microtome equipped with a Diatome diamond knife were routinely stained with uranyl acetate and Reynold´s lead citrate and then observed with a Zeiss EM 900 transmission electron microscope operated at 50 kV. Most images were recorded digitally. Semi-thin sections (1.5-2 μm) were stained with 1% toluidine blue and examined by light microscopy.

Negative staining: To visualize the virus particles associated with the reproductive system of *C. flavipes* females, the organ was macerated and the homogenate passed through a 0.45 μm filter. The filtered virus particles were absorbed onto a 300 mesh Formvar-coated

Figure 1. Transmission electron (TEM) micrographs of sections of the ovary and calyx of *Cotesia flavipes*. **A.** Oocytes (**oo**) surrounded by follicular cells (**fc**) in the lower ovary, with no signs of virions in the cell cytoplasm or lumen of the ovariole. **B.** Section of the calyx region of a *C. flavipes* ovary showing the chitin layer (**arrowheads**) isolating the calyx epithelium (**ce**) from the virus-producing calyx cells (**cc**). **C.** A large vacuole filled with virions (*****) is present in the cytoplasm of a calyx cell (**cc**). **D.** The virus-containing vesicles migrate to the edge of the calyx where the virions are unloaded. Arrowhead indicates an opening in the calyx cell that allows the release of the virions. The inset shows a vesicle preparing to release virions at the edge of the lumen. **E.** Lumen (**lu**) of the lower calyx filled with virions surrounding the developing oocytes (**oo**). **F.** A close-up of **E** showing that only cells of the calyx epithelium (**ce**) remain. **G.** Detail of the surface of the egg chorion (**ch**) and the amorphous layer (**arrowhead**) that keep the virions away from the microvillus-like projections of the chorion. **H.** Detail of CfPDV virions from the calyx lumen. The **arrow** indicates the tail-like structure of the virion and the **arrowhead** indicates a nucleocapsid. **I** - Overall structure of the virion envelope and its tail after negative staining. Inset shows virions in detail. Note the tail-like structures. (**cc** - calyx cell, **ce** - calyx epithelium, **ch** - chorion, **fc** - follicle cell, **lu** - lumen, **mc** - muscular cell, **n** - nucleus, **oo** - oocyte, **v** - virion).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nickel grid for 1-2 min, quickly washed in distilled water, treated with 1% uranyl acetate for 1 min and examined in a Zeiss EM 900 transmission electron microscope operated at 50 kV.

PCR analysis. Template DNA from dissected ovaries of *D. areolatus* and *C. flavipes* (n = 5) was prepared by extraction in TEN buffer (10 mM Tris-HCl pH 8.0, 2 mM EDTA, pH 8.0 and 0.4 M NaCl) added of 40 µl of 20% SDS and 8 µl of 20 mg/ml proteinase-K. After samples had been incubated in a water bath at 55°C for 1 h, 300 µl of 6 M NaCl was added, followed by vortex mixing for 30 s and centrifugation (14,000 *g* for 30 min). After centrifugation, the supernatants were transferred to new vials, an equal volume of ice-cold 100% ethanol was added and samples placed at -20°C for 1 h to precipitate the DNA. After precipitation, samples were centrifuged (14,000 *g* for 20 min at 4°C) and pellets were washed once in ice-cold 100% and twice in 70% ethanol, followed by air-drying for 15 min [1]. The confirmation of infection by *Wolbachia* was done by amplifying the *wsp* gene using the specific primers 81F (5′-TGGTCCAATAAGTGATGAAGAAAC-3′) and 691R (5′-AAAAATTAAACGCTACTCCA-3′), with water as the template for the negative control. The reactions involved an initial incubation at 95°C for 2 min followed by 35 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 2 min, with a final extension at 72°C for 10 min after the last cycle [55]. The PCR products were resolved on a 1.5% agarose gel in TAE buffer, stained with ethidium bromide and viewed on a UV transiluminator.

RESULTS

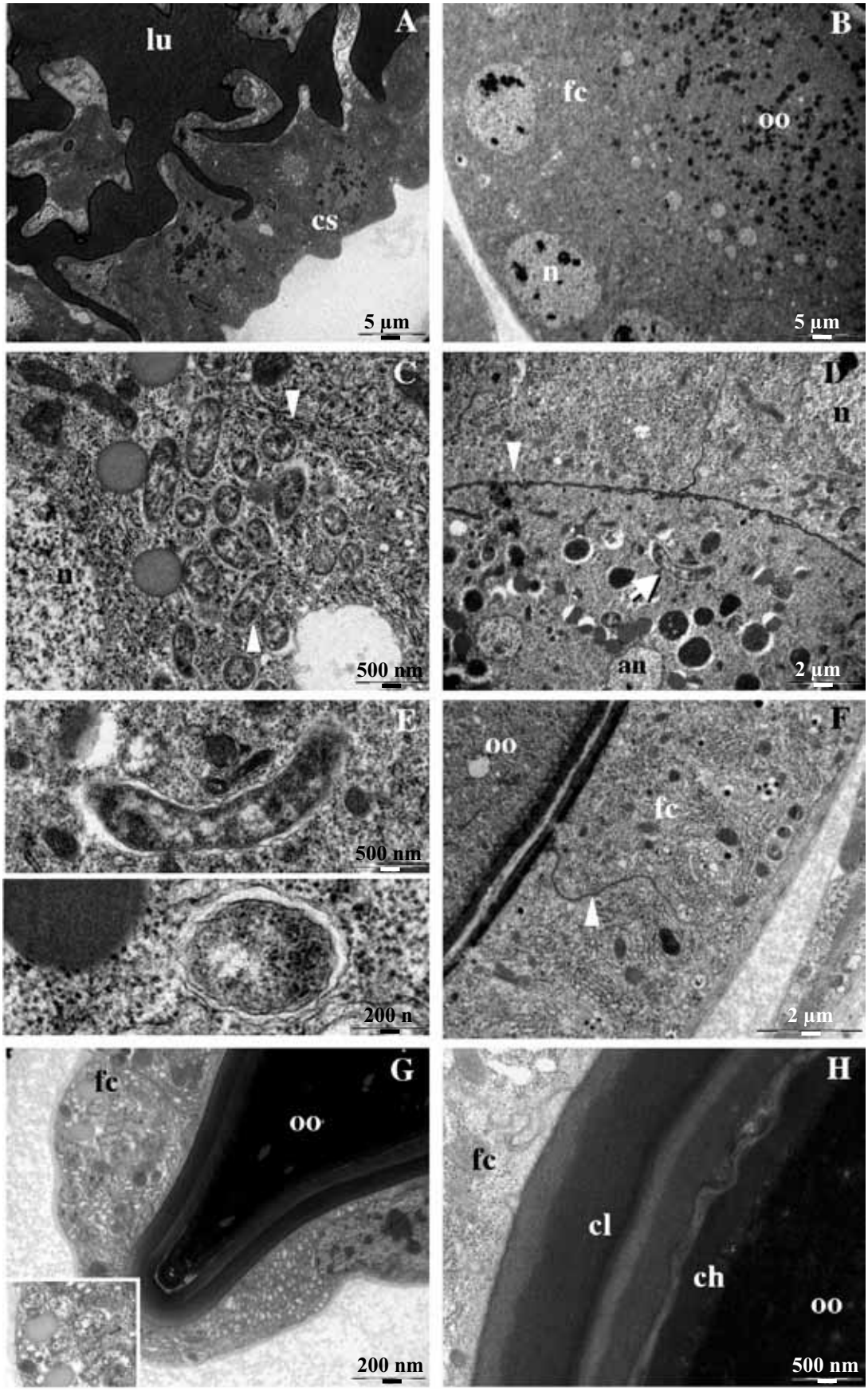
Cotesia flavipes and *D. areolatus* had different symbionts associated with their reproductive systems since virus particles were found in *C. flavipes* whereas *Rickettsia*-like bacteria were the only symbionts found in the reproductive system of *D. areolatus.*

The virus particles (CfPDV) associated with the reproductive system of *C. flavipes* were abundant in the cells and lumen of the calyx and in the oviduct surrounding the developing oocyte (Fig. 1). No virus particles were detected in the lumen of the lower ovary or in the follicular cells surrounding the developing oocytes (Fig. 1A), probably because of the age of the females used in this experiment (2-4 d old) since most of the replication occurs late in the pupal stage or early in adult development [10,54]. Although cells in which viral replication could occur were not seen in the females used here, a number of virus-containing vesicles were present in the cytoplasm of calyx cells and were isolated from the calyx epithelium by a thin cuticle layer (Fig. 1B,C). The virus particles in these vesicles were seen being unloaded into the calyx lumen close to the surface of the oocyte (Fig. 1D). As virions were produced and unloaded as the developing oocyte would progress into the calyx towards to the oviduct a large number of virions were observed in the lumen, with a progressive thinning and disintegration of the calyx cells (Fig. 1E,F). In the calyx and oviduct, the virions were isolated from the outermost microvillous-like projections of the parasitoid egg chorion by an amorphous layer (Fig. 1G). No virions were seen physically attached to these chorionic projections, even lower down in the lateral oviduct.

Negatively stained CfPDV from filtered ovarian extracts and ultrathin sections of *C. flavipes* ovaries were pleomorphic, ranging from 110 to 300 nm in length and 70 to 190 nm in width, with a tail-like structure that could be twice as long as the envelope (Fig. 1H,I). The virion envelope consisted of a single membrane encasing 1-10 cylindrical nucleocapsids that appeared as dense structures, usually with a circular or rectangular profile 30-40 nm in diameter.

No virus-like particles were detected in the ovary or venom gland of *D. areolatus* (Fig. 2). However, this parasitoid was shown to harbor *Rickettsia*-like bacteria in the ovary, particularly around the nuclear envelope of follicular cells (Fig. 2B,C) and in the developing oocyte (Fig. 2D,E). The

Figure 2. TEM of the ovary and venom gland of *Doryctobracon areolatus*. **A.** Venom gland showing large secretory cells (**cs**) and a ramified lumen (**lu**). **B.** Bacteria surrounding an oocyte (**oo**) in a follicular cell (**fc**); **C.** Close up of a follicular cell infected with bacterial cells. Note the bacteria (**arrowheads**) concentrated around the nucleus (**n**). **D.** A bacteria-infected oocyte. Note the bacterium (**arrow**) close to the vitellinic membrane and the accumulation of an electron-dense matrix (**arrowhead**) at the interface of the chorion and follicular cells (**an** - accessory nucleus of the oocyte). **E.** Close up of two bacteria showing the double membrane of the cell wall and an outer layer typical of *Wolbachia* cells. **F.** An oocyte halfway to the ovary. Note that the follicular cells (**fc**) are still very large and there is an accumulation of electron-dense matrix attached to the cell surface; this matrix isolates the cells from the chorionic layer of the developing oocyte (**oo**). (**Arrowhead** indicates a septate junction separating neighboring cells). **G.** A mature oocyte (**oo**) showing the thick layer that isolates it from the follicular cells; the latter show signs of degradation seen as numerous vesicular bodies in the cytoplasm. Bacteria were also seen in cells at this stage of development (inset shows bacterial cells in detail). **H**. A close up of the thick coating layer (**cl**) separating the egg chorion (**ch**) from the follicular cells (**fc**).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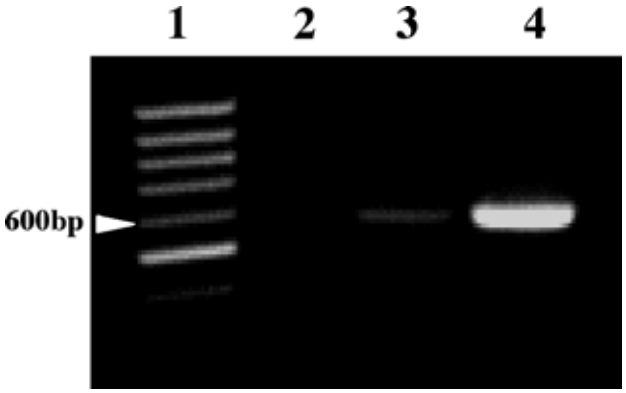

Figure 3. PCR analysis to detect *Wolbachia* in *Doryctobracon areolatus* and *Cotesia flavipes* ovaries. Different samples were loaded in each lane: 1 – 100 bp DNA ladder, 2 – negative control (water), 3 – *D. areolatus* gDNA sample 1, 4 – *D. areolatus* gDNA sample 2, 5 – *C. flavipes* gDNA sample 1, 6 – *C. flavipes* gDNA sample 2.

bacteria were abundant in but not evenly distributed among follicular cells. Regardless of whether they were infected or not, the follicular cells remained around the developing oocyte until they reached the end of the lateral oviduct. At this point, these cells were much smaller but still active since they seemed to synthesize a thick layer of varied electron density that covered the egg chorion (Fig. 2F-H). The bacteria found in the ovary and eggs of *D. areolatus* were pleomorphic, ranging in size from small rod-like/coccoid cells to very large forms (Fig. 2C,E). Several factors, including the occurrence of bacteria in the egg cytoplasm and the bacterial cell morphology, e.g., the presence of three enveloping membranes, the double membrane of the cell wall and the presence of an outer layer derived from the host, were typical of *Wolbachia* [20]. The identity of *Wolbachia* in *D. areolatus* tissues was confirmed by PCR using specific primers for the *wsp* surface protein (Fig. 3).

DISCUSSION

Although several new viruses have been reported to be associated with insect parasitoids, polydnaviruses are obligatorily associated with Braconidae and Ichneumonidae, and are classified into two genera (Bracovirus and Ichnovirus). Bracoviruses occur in the subfamilies Cardiochilinae, Cheloninae, Microgastrinae and Miracinae of Braconidae, whereas ichnoviruses occur in three subfamilies of Ichneumonidae (Campopleginae, Ctenopelmatinae and Banchinae) [38,44].

The morphology of the most abundant virus particles seen in *C. flavipes*, which belongs to the subfamily Microgastrinae, was essentially similar to that of bracoviruses from other braconids. The virions were ovoid and contained several electron-dense nucleocapsids enclosed by a single unit membrane envelope. The number of nucleocapsids in virions of CfPDV was variable (up to 10) and they were similar in size to other bracoviruses [11,16]. The number and size of nucleocapsids in PDVs are species-specific, with these parameters being positively related to the size of the DNA segments that they enclose. The general morphology of the CfPDV, including the tail-like structure of the nucleocapsid, was similar to the bracovirus associated with *C. congregata* [10]. However, CgPDV is not the only virus particle associated with *C. congregata.* Buron and Beckage [10] reported the presence of a virus-like filamentous particle replicating in the nuclei of cells in the upper calyx of this parasitoid. Virions were reported associated with the cytoplasm of these cells, but free particles were seldomly seen in the lumen of the calyx [10].

The amorphous layer found on the egg surface of these braconids may correspond to the immunoevasive proteins secreted into the oviduct and that cover the egg surface to facilitate host colonization [3,15,42]. The convoluted chorionic structure of hydropic eggs has been related to the requirements of the yolk-poor eggs to grow as they absorb nutrients from the host to sustain their own embryonic development [33,45]. Although microvillous-like projections have been described in endoparasitic species harboring symbiotic virus particles that physically anchor onto these projections [12,42,49], the CfPDV particles were never seen in close contact with the chorionic surface, even when near the end of the common oviduct.

The absence of PDV-like particles in *D. areolatus* was expected since there are no reports of PDV associated with braconids of the subfamily Opiinae [38,44]. The population of *D. areolatus* examined here also had no non-PDV found in other braconid subfamilies, including the Opiinae *Diachasmimorpha longicaudata* (Hymenoptera, Braconidae) [23,24]. *Diachasmimorpha longicaudata*, a parasitoid of fruit flies, carries an entomopoxvirus in its venom apparatus that is injected into the host hemocele to disrupt the normal function of host hemocytes, thereby ensuring successful colonization by the parasitoid larva [24].

In contrast, *D. areolatus* harbored the *Rickettsia*-like bacteria *Wolbachia*, a cytoplasmic symbiont

associated with a large number of arthropod species and that has a natural infection rate of 15-20% in insects [39,53]. *Wolbachia* can be transmitted vertically and horizontally and can manipulate host reproduction through parthenogenesis, male feminization and cytoplasmic incompatibility [51]. *Wolbachia* may exert different effects on the host, depending on the bacterial strain and on the tissue infected.

In terms of the physiological costs parasitoid females may have by carrying such symbionts, these bacteria may be beneficial to its host by increasing host survivorship and tolerance to stress or detrimental by reducing overall fitness, but there are no reports of *Wolbachia* influencing any physiological process of host-parasitoid interactions [17,32,36,41,50]. Nevertheless, *Wolbachia* is a symbiont that has been extensively studied for the development of strategies to control insect pests, especially mosquitoes [35]. In particular, the *in vitro* culturing and artificial transmission of these bacteria may provide new insights into how this microorganism can be used in the management of insects [19,29].

In conclusion, the finding that only a single virus is associated with *C. flavipes* will facilitate the isolation and characterization of the genome of polydnaviruses associated with this wasp species. This simple relationship should also facilitate the discovery of virus-produced molecules that may be potentially useful in regulating insect growth and in developing biologically-based methods of pest control.

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