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Developmental Biology among Corbiculate Bees: *Bombus impatiens,* Including Observations on Its Egg Eclosion

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ABSTRACT

This contribution results from an investigation of four commercially obtained colonies of *Bombus (Pyrobombus) impatiens* Cresson in an attempt to understand the functional anatomy and behavior of its immature stages. Eggs are described in detail, and their chorionic microstructure is contrasted with that of *Bombus (Cullumanobombus) griseocollis* (De Geer). They are deposited in groups consisting of a few to more than nine eggs into a single chamber. The study confirms that larvae pass through four instars. Although increasing in size dramatically from one instar to the next, larval anatomy and behavior change little during the first three instars. The last instar is the one that commences defecation and production of silk resulting in cocoon construction. In contrast to most bees, its larval activities result in substantial changes in size and shape of its brood chamber, whereas brood chambers of most bees are constructed by the female and modified little by the offspring. This study is a part of a series of investigations into the similarities and differences in the developmental biologies among corbiculate bees.

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INTRODUCTION

Michener (1953) presented a detailed comparative account of available mature larvae of bees and followed that study with a similar but less complete investigation of pupal bees (Michener, 1954). Since then, other bee specialists, including J.G.R., have attempted to broaden humanity's understanding of the immature stages of bees by collecting and studying additional taxa. Although J.G.R. has reported on euglossine bees (Garófalo and Rozen, 2001; Rozen, 2016a; Rozen, 2018), his focus has been primarily on the immature stages of noncorbiculate solitary and cleptoparasitic bees, in part because their biologies were understudied relative to those of social bees and in part because his geographic location was removed from areas with a fauna of stingless bees. However, his recent investigation of the immature stages of *Bombus* (*Cullumanobombus*) *griseocollis* (De Geer) with C.S.S. and Dennis E. Johnson (Rozen et al., 2018) has now attracted his attention to corbiculate bees in general. Although the current project again investigates the genus *Bombus*, other studies are planned pertaining to stingless bees and, it is hoped, to other euglossines. *Bombus* (*Pyrobombus*) *impatiens* Cresson has been selected for this paper because it is commercially available throughout the year, permitting inquiry during inclement winter conditions since these animals can be maintained and reared in the laboratory.

We present new insights into the nesting biology of this species based on observations of its nests and information concerning immature stages. The life stages (eggs, larval instars, pupae) of *B. impatiens* are morphologically described and illustrated. The reader should understand that this presentation does not refer to other species of *Bombus*, for there is a vast amount of literature referring to them. As indicated by the title, the intent of this paper is to focus on this species; comparison with other species will come later. Although we have an excellent overview of the biology of the genus (Michener, 2007) and even lengthy treatments of various aspects of the biology of *B. impatiens* in particular (Plath, 1922, 1927, 1934; Frison, 1929; and Townsend, 1951), there is little reference to developmental biology. Recently Cnaani et al. (2002) presented certain important details of the developmental biology of *B. impatiens,* but unfortunately that study was undertaken before SEM investigations were widely available. Accordingly, it did not deal with the subject of egg eclosion, which, as shown below, may be a future fruitful path of inquiry.

Background on Egg Eclosion in Bees: The first reference to egg eclosion in bees was that of *Svastra obliqua obliqua* (Say) (Apidae: Eucerini) in which the hatching first instar covered by chorion was diagramed as the chorion split along the spiracular line (Rozen, 1964: fig. 5). More recently, it was determined, from field sketches made in 1968 of the egg of *Epicharis albofasciata* Smith (Apidae: Centridini), that while hatching its chorion also split along the spiracular line on both sides as the first instar emerged (Rozen, 2016b: figs. 16, 17). Torchio and Trostle (1986: fig. 2) photographed the same phenomenon in their study of *Anthophora urbana urbana* Cresson (Apidae: Anthophorini). However, only recently has the SEM revealed the presence of small, sharp pointed spicules that are involved with the splitting of the chorion. With many of the taxa studied, the spicules formed a band that ran along both sides of the first

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instar just above the spiracles (e.g., Rozen et al., 2006: figs. 14, 15; 2011a; figs. 15–18). Because the path was the same as the chorionic split occurring as the first instar started to emerge, there was no doubt that the spicules are involved with the renting of the chorion and thus were termed "hatching spines." These have been identified in many groups of bees: see Rozen et al., 2017: table 1. Therein it was hypothesized that these small, sharp-pointed spines that occur just above the spiracular line on the two sides of the developing instar are involved with the distribution of a hatching enzyme that results in the splitting of the chorion along both sides of the body, thereby freeing the first instar in many taxa. However, as revealed in this current study, no such double row of spines have been discovered in the case of *B. impatiens*, but as shown below, there are spines radiating around each of the first instar's spiracles that do indeed have a function that will almost certainly be found to be involved with ecdysis.

MATERIAL AND METHODS

Four nests of *B. impatiens* were dissected and examined. Nest 1 was obtained from Koppert Biological Systems, Howell, Michigan. It had been obtained by Princeton University several weeks before it was dissected and initially examined by us on 29 November 2017. Its yield consisted of a single cluster of seven eggs and many larvae and seven pupae. Nest 2 was obtained from Biobest, Romulus, MI. It was initially examined 11 January 2018 at the American Museum of Natural History by J.G. Rozen and C.S. Smith. Although the queen and most workers had expired during shipment, presumably because of unusually low temperatures, this nest provided many brood chambers containing eggs and live young instars as well as last larval instars and pupae. Subsequently, Biobest provided a replacement colony, Nest 3, which arrived on 16 January 2018. It was kept active to observe nesting activities of workers and queen. Larval specimens from Nests 1 and 2 are identified by dates of the first specimens obtained from each of the nests. After studying the first three nests, yet another nest (Nest 4) was ordered from Koppert. This was necessary to garner illustrative material to demonstrate what we learned about nest features from dismantling the previous nests.

During this investigation, collected eggs, larvae, and pupae were killed, preserved, and then stored all in Kahle's solution. SEM micrographs were captured with a Hitachi S5700 in the Microscopy and Imaging Facility of the American Museum of Natural History. Microphotographs were taken with a Canon Power Shot A2300 camera hand held to one of the eyepieces of a Leitz Wetzlar stereomicroscope or a Carl Zeiss compound microscope.

OBSERVATIONS ON NESTING BIOLOGY

Number of Larval Instars: To determine the number of larval instars that are involved in the development of this species, widths of larval head capsules recovered from nests 1 and 2 were measured. Thus, table 1 presents head widths of 55 larvae of *B. impatiens* collected from 11 brood chambers lacking cocoons from Nest 1. Among last larval instars, only predefecating forms were included. To ascertain that these were the final larval instar, head widths of three

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TABLE 1. Mean larval head widths and ranges (in mm) of *Bombus impatiens*, demonstrating four larval instars. Number of data in parentheses. Gap = distance in mm separating means.

Nest 1		Gap		Gap		Gap	
Instar	1		2		3		4
Mean	0.65 (16)	0.26	0.91 (15)	0.32	1.23 (15)	0.48	1.71 (9)
Range	0.6–0.7		0.85–0.98		1. –1.36		1.63–1.
Nest 2							
Mean	0.69 (18)	0.18	0.87 (31)	0.29	1.16 (14)	0.53	1.69 (32)
Range	0.65–0.73		0.8–0.93		1.15–1.25		1.5–1.85

postdefecating larvae removed from cocoons were added, as shown in figure 1, to demonstrate their similarity in size with those without cocoons. While these three larvae had slightly larger head widths, the differences between the two are within the range of variation both of the last instars and of the pupal sizes exhibited in preserved specimens. One larva with a head width of 1.1 mm seemed somewhat intermediary between second- and third-instar categories. However, the specimen clearly exhibited small, paired dorsal thoracic tubercles immediately identifying it as a third instar. In Nest 2 (table 1) all available head-capsule widths were recorded, including many of last larval instars removed from cocoons. On the basis of four discrete peaks in size (fig. 1), we confirm that *B. impatiens* undergoes four larval instars.

The Nest: When viewed from above (fig. 2), Nest 4 consisted of five major groupings of closed chambers identified with circled numbers and two groupings of empty cells that would remain open and be used by worker bees primarily for honey storage. Also present were some other small groups of chambers (e.g., figs. 3, 4) that contained young larvae. Among the major groups, Group 1 cells contained last larval instars as identified by fecal blotches showing through the surface of cocoons. Arrows toward the bottom of Group 1 point to an elongate chamber split open to reveal young instars, and to a small closed chamber with young larvae. Group 2 consisted of a basal group of cocoons with grayed fecal blotches at base, with less gray (younger) cocoons above that were partly covered by seven orange-toned enclosed egg chambers (arrows). Group 3 contained numerous cocoons and a single small, orange-toned chamber (arrow) enclosing a cluster of eggs. Group 4 contained only last larval stages. Group 5 consisted of a large group of fourth instars most of which were found to have started cocoon spinning. Figure 5 is a close-up of four (arrows) of the opened egg chambers identified by rectangle in fig. 2.

All of these egg chambers in Nest 4 that were opened for investigation were reclosed within 30 minutes by worker adults when the workers were reintroduced into the nest. Furthermore, a last-stage larva that accidently had its cocoon partly torn earlier was recovered by these adults. Not visible in Nest 4 (fig. 2) was a small oblong chamber attached to the plastic substrate of the shipping cage. When opened (fig. 6), it was found to contain 42 small larvae, mostly second instars but also a few third instars, all within the single chamber making it the most densely populated brood chamber detected in the study. With them were a number of fractured masses of agglutinated pollen (arrows) on which they had obviously been feeding. After it was

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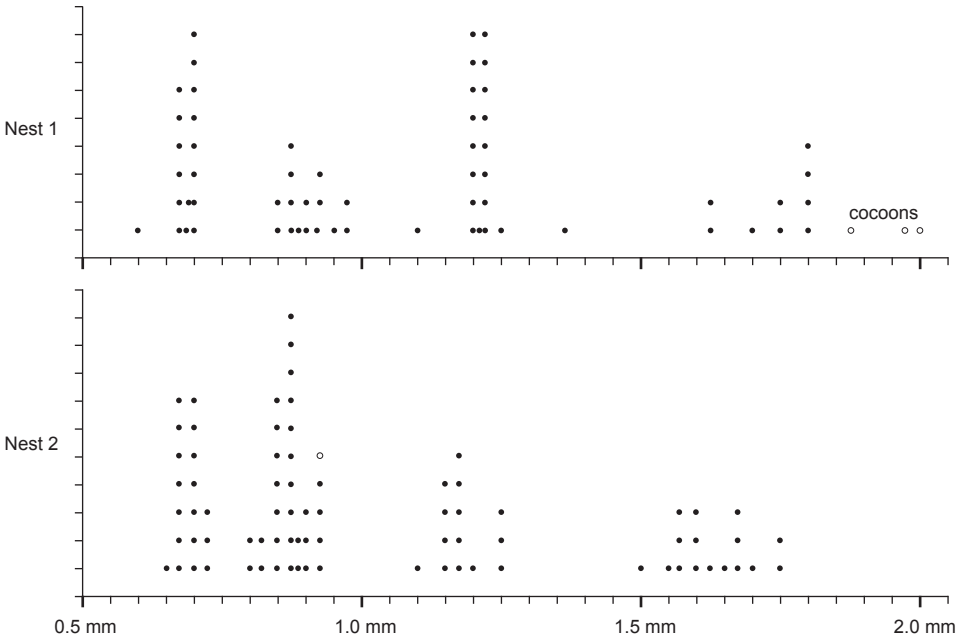

FIGURE 1. Diagram plotting head widths of all larval specimens collected and preserved from Nest 1 and Nest 2 demonstrating that this species has four larval instars based on the occurrence of four distinct size classes.

realized that workers resealed egg chambers when inspected chambers were reintroduced into the colony, a Petri dish containing the fragmented chamber and 42 larvae were placed the same day within the colony at 2:10 PM. By 2:33 PM the workers had resealed the chamber (fig. 7), excluding only one larva, shown here.

Brood Chamber Modification: All brood chambers populated with eggs or young larvae (instars 1, 2, and 3) contained more than one immature. Table 2 lists the number of these immatures found per chamber in each of the two nests as well as totals. Last larval instars commence to divide jointly occupied chambers as soon as they start cocoon spinning, resulting in each larva surrounded by its own cocoon, thereby its own brood chamber.

With most bees, the term *brood cell* refers to the nest structure constructed by the female that she uses to hold provisions and one egg that subsequently develops into a larva. Although an offspring may or may not later wrap itself in a cocoon, it does not modify the cell in other ways. Because the developing offspring of *B. impatiens* strongly modifies its surrounding structure as it grows, the structure is here usually termed a *chamber*. With *B. impatiens* and probably most other nonparasitic *Bombus* species, the queen or workers form a small waxen cup into which eggs are deposited, and the cup is closed over with an orange-toned wax cover (in figs. 6, 7, the tops of the cups were spread apart with forceps to reveal egg clusters). The so-called wax is soft, pliable, and may include some pollen in addition to wax. As the resulting larvae grow, the wax cup increases in size, presumably because workers add wax. With this species, food is probably injected into the chamber with the proboscises by attending worker bees (a task presumably performed by the queen for the first generation of workers). The increase in

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TABLE 2. Number of eggs and young larvae (instars 1–3) per chamber recovered from brood chambers in nests 1 and 2.

Number of Offspring	Nest 1	Nest 2	Total
3	3	–	3
4	2	–	2
5	2	1	3
6	2	4	6
7	2	4	6
8	1	1	2
9	–	1	1

larval size results in an increase in the size of the chamber but does little to reshape the outer surface of the chamber. However, probably toward the end of the development of the third instars or the start of the fourth gradual modification of the outer surface of the chamber takes place. At that time the outer surface over each larva in the chamber begins to bulge (fig. 12), suggesting that the size of each contained individual influences the form of the covering as well as the volume of the chamber.

With the start of the fourth stadium, each larva starts to produce and surround itself with fine silk. Silk is extruded in curly strands forming a soft, cottony, pillowlike cushion of silk (fig. 16) separating the larva from the outer waxen chamber surface, which is sufficiently soft that it can be easily penetrated from the outside by food-supplying proboscises of adults (or penetrating forceps). Silk produced by adjoining larvae form separate cocoons (figs. 12–15). The growth of each cocoon is directed outward, away from their clustered bases, as the larvae feed and grow while slowly curling and uncurling. Thus, they press their cushionlike cocoons against the soft outer waxen wall causing it to bulge. As a result, what was originally a small wax-covered egg chamber becomes a multidomed cluster of individual cocoon chambers, created mostly by the larvae themselves. There is no compelling evidence that workers applied wax to the outer surface of the cocoons as the larvae increase in size and expand the outward growth of the cocoon chambers. The exposed surface of the cocoon gradually lightens in color, becoming whitish because of silk showing though the thinning wax covering. Since larvae within the cocoon commence defecation during silk production, some opaque wax-colored fecal pellets become incorporated and flattened in the cocoon fabric and, because of their dark opaqueness, can be seen from outside. They gradually darken further and become a dark gray-brown, so that an observer can usually distinguish relative ages of cocoon clusters in a nest.

Cocoons are ovoid in shape, and several had diameters of 8–9 mm and lengths perhaps 2× their diameters. Other cocoons in a nest have both larger and smaller diameters. Distinct fenestrations in the cocoon surface were not certainly observed but, if present, might appear to be clogged by liquid honey.

Although the fecal pellets are visible in the cocoon fabric of completed cocoons, cocoons from which adults have emerged seem to have incorporated much of the fecal mass inside at their base. Because last larval instars were observed starting defecation after a network of cocoon silk had been deposited, defecation does not precede initiation of cocoon spinning with this species. The somewhat uneven, very dark, inner surface of the feces at the bottom of several vacated chambers appeared covered by a thin, glistening transparent surface of cellophane-like silk upon which rested the opaque-white crumpled caste pupal exoskeletons (fig. 17, arrows). Cocoon fabric appears to exhibit no special organization, and there is no configuration or structure that would indicate a special passageway for exchange of air between cocoon

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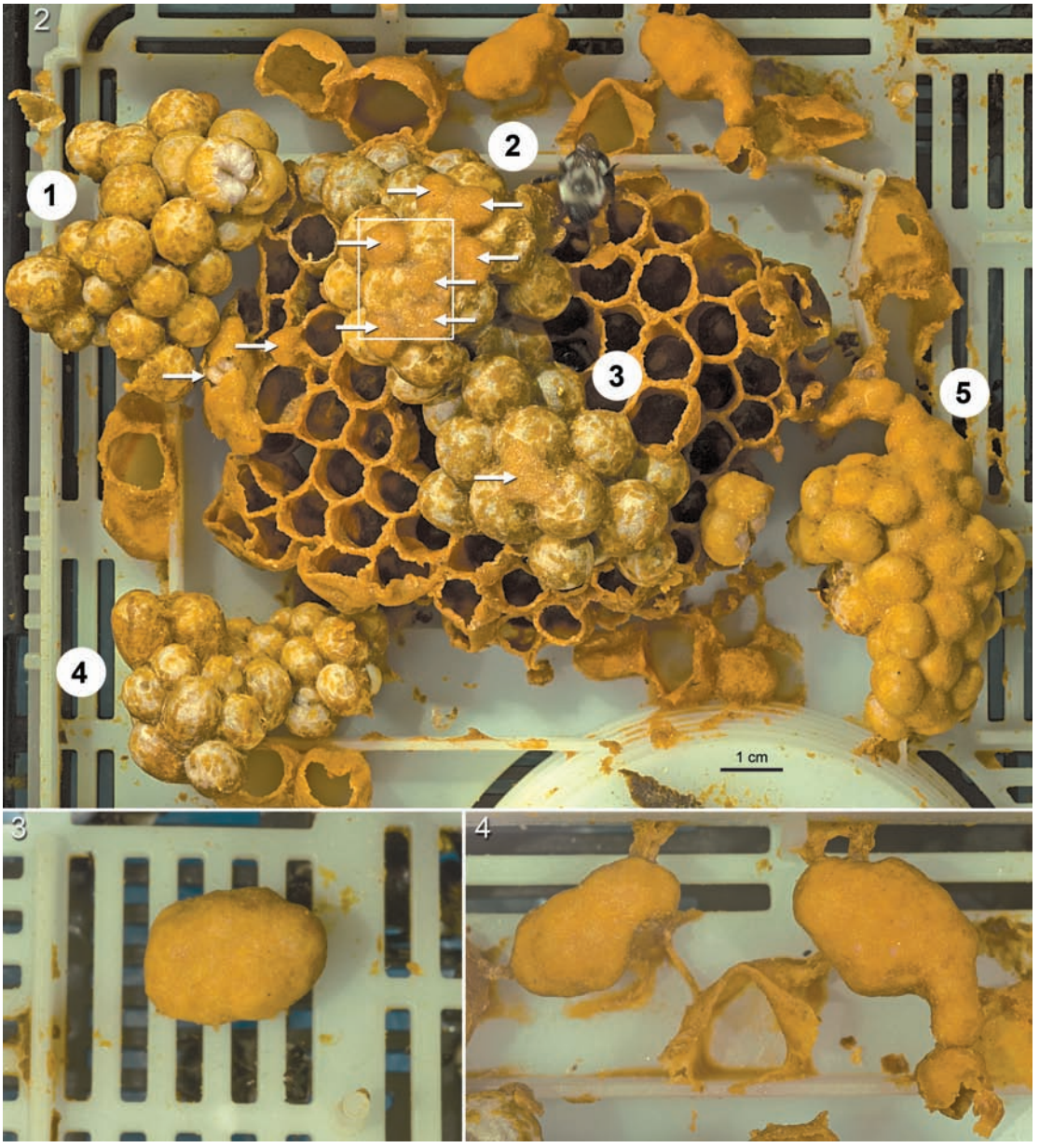

FIGURES 2–4. **2.** Top view of most of Nest 4 consisting of five major groupings of closed cells identified by circled numbers and two groupings of empty cells. **3, 4.** Also present were a number of the small groups of chambers containing young larvae.

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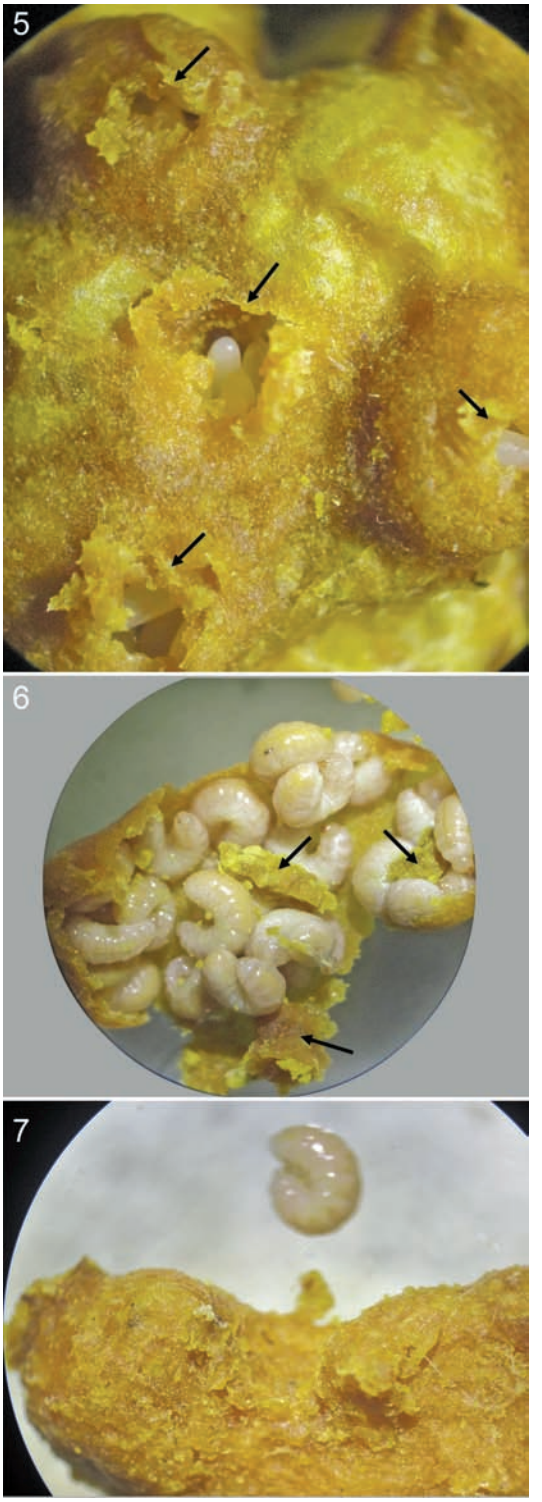

lumen and exterior such as has been detected in other groups of cocoon spinning bees (Rozen et al., 2011b).

Egg Eclosion: Eggs of *B. impatiens* are described below in the section on Descriptions of Immature Stages. Here we describe their hatching process. The sample of first instars from Nest 2 included a number of individuals that still carried remnants of their partly shed chorions on the posterior ends of their bodies (e.g., figs: 23, 25, 30). In all these cases, the first instars exited or were exiting through the front end of the chorion, and none of the chorions were split along the sides of the larva's body, as further discussed below. There was no evidence of an early instar. When first instars were examined with an SEM, they revealed no suggestion of linear row of hatching spines along each side of the first instar's body (figs. 26, 30), as has been recorded on first instars of taxa in certain tribes other than Bombini (Rozen et al., 2017). However, they did reveal rosettes of fine, outward radiating spines surrounding spiracles that we now interpret as part of the hatching process (see below). Thus, it seems that the larva slowly emerges forward from its egg shell by squeezing its body through the long tube of somewhat firmer, reticulated chorion along the length of the egg while the smooth, transparent chorion at the rear of the egg collapses thereby avoiding a vacuum resistance behind the larva and the end of the chorion, as has also been suggested for *B. griseocollis* (Rozen et al., 2018).

Slightly wobbling body motions were often apparent on live specimens while the chorion

FIGURES 5–7. **5.** Close-up of four (arrows) of the egg chambers opened for examination in Group 2. **6.** Part of opened oblong chamber containing total of 42 young larvae (arrows pointing to food). **7.** Same chamber as in figure 6, all now rewrapped by workers except for one larva.

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still covered the anterior end of eggs about to hatch. Eggs in a chamber tended to develop more or less synchronously, as might be expected since the queen populates a chamber with eggs one after the other.

While this still seems to be a reasonable explanation regarding the function of the clear flexible chorion at the rear of the egg of *B. impatiens*, many of the specimens shedding their chorions presented unanticipated information: alteration of the chorion coincides with the position of the spiracles of the pharate first instar (figs. 19, 24, 28, 30). The alteration involved the loss or fracturing of chorionic reticulation immediately over the position of the spiracle, revealing either the smooth, opaque underlying serosa or a complete aperture of the chorion and serosa overlying the spiracle. Thus, with some specimens these modifications were revealed as a linear series of small local chorionic disruptions along the side of the pharate first instars, although they were not revealed in any that showed a longitudinal split in the chorion as, for example, in *Epicharis albofasciata* Smith (Rozen, 2016b: fig. 16) and a good many other hatching bees. What causes these modifications and what role do they play in eclosion?

The following hypothesis responds to these questions. The fact that these apertures are associated with the spiracles suggest that they may be created by a hatching enzyme emitted from the spiracles, as proposed in Rozen et al., 2017. In this case, the enzyme does not promote a splitting of the chorion but only an opening allowing respiration during the time that ecdysis slowly takes place. It would probably be unnecessary for all spiracles to be exposed to the atmosphere, presumably making the period before the unveiling of the first thoracic spiracle the most critical, since before that time tracheal respiration would not be possible. Might the somewhat larger opening of the first pair of spiracles (figs. 26, 32) be evidence of their importance to the emerging larva? The presence of sharply pointed, radially projecting spicules surrounding spiracular openings displayed on specimens (fig. 26) initially suggested that they might perhaps be involved with fragmentation/dissolution of the amniotic membrane in connection with the splitting of the chorion along both sides of the egg, as has been suggested on those species that have a linear row of spines above the spiracular line in some other taxa (Rozen et al., 2017). However, this seemed unlikely since the chorion of *B. impatiens* does not split. Furthermore, when the chorion of a hatching egg (fig. 33) was accidently broken during collection, leaving the serosa-covered first instar exposed (fig. 34), it became apparent that the widely spaced small spiracles surrounded by rosette spines were too distant from one another to result in chorion splitting between spiracles. When viewed closely (fig. 27), the serosa-covered radiating spines on each rosette were more or less decumbent, and those on opposite sides of a central depression (the serosa-covered atrial opening, e.g., fig. 39) pointed away from one another. This suggested that the serosa covering the atrial opening might be tautly held in place by them, so that as the tracheal system expels the hatching enzyme under pressure, the serosa covering each spiracle ruptures. The resulting rent in the serosa is visible afterward (arrow, fig. 39). When the enzyme is released, it destroys the chorion immediately over the spiracle and in some cases (figs. 30, 31) the thin basal chorion layer behind the spiracle is also destroyed as the hatching larva slowly moves forward out of its chorion. This hypothesis implies

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that the serosa still covers the first instar and does not explain how it is removed, although some views show that it is extremely thin and easily fragmented (fig. 22).

DESCRIPTIONS OF IMMATURE STAGES

Eggs

Figures 8, 11, 18, 41–49

Description: Color white. Form (figs. 8, 41) moderately robust, slightly curved with anterior end (identified by position of micropyle) somewhat more rounded than more pointed posterior end. Chorion of large middle part of egg somewhat thicker, ridged, and strongly reticulate as seen on dried specimen (fig. 41), only moderately reflective, while chorion at extreme anterior and posterior ends thin, flexible, more transparent though faintly textured; borders of these two surfaces identified because of contrasting chorionic thicknesses and texturing (figs.11, 18); flexible chorion at front end considerably less extensive compared with that of *B. griseocollis*, appearing in lateral view confined to front surface of egg; flexible chorion at rear perhaps less extensively reduced compared with that of *B. griseocollis*. Micropyle difficult to identify with stereoscope but clearly visible with SEM on anterior end of egg as tight cluster of holes surrounded by radiating chorionic ridges (figs. 42–44).

Material Studied: Nest 1: Average length of egg 3.58 mm, range 3.35–3.90 mm; width range 0.9–1.05 mm (n = 3). Nest 2: Average length of egg 3.32 mm, range 3.2–3.4; width range 0.9–1.1 mm (n = 21). Eggs produced by workers from Nest 3: Average length 2.88 mm, range 2.7–3.0 mm; width range 0.8–1.0 mm (n = 8). Eggs produced by workers from Nest 4 measured live: Average length 2.86 mm, range 2.7–3.5 mm; width range 0.75–1.0 mm (n = 25). Eggs measured from Nests 1 and 2 were produced by queens and were therefore fertilized, so would have been female. Those from Nest 3 and 4 were produced by workers and were male. Unanswered is whether this difference in size is an innate sexual difference or the result of queens having a larger body than workers.

Comparison of Chorions of *Bombus impatiens* and *Bombus griseocollis*: While first examining the preserved egg of *B. impatiens*, J.G.R. noted that the chorions of this species seemed less ridged and more pliable than those of *B. griseocollis*, which he had recently examined (Rozen et al., 2018) as had Stephen and Koontz (1973). At the time, he gave little thought to these features since preserved specimens were often unreliable in evaluating such matters as they occur in life. However, detailed study of the similarities and differences in chorions of the two species probably explain differences in chorionic flexibility. Eggs of the two species certainly share striking similarities in general appearance with their two ends covered by thin transparent flexible chorion separated by a long stretch of densely reticulated chorion. Both reveal that the reticulations are similar in that they result from connecting mostly hexagonal ridges that arise from a thin basal chorionic surface. However, they differ significantly, as follows:

In *B. griseocollis* connecting hexagonal ridges rise above the thin basal sheet of chorion to their maximum length thereby creating a thick, ridged outer framework, providing resistance

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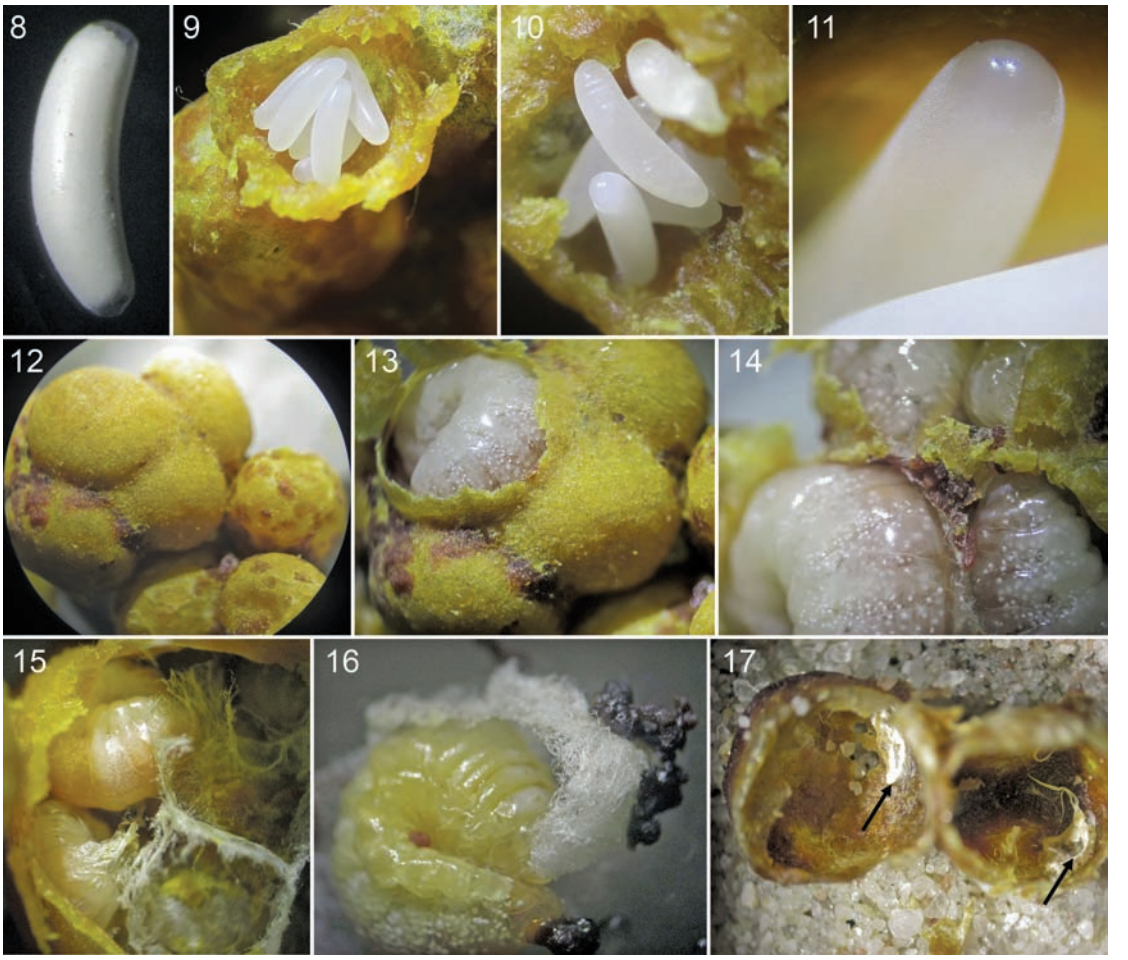

FIGURES 8–15. Microphotographs of Nest 3. **8.** Egg, anterior end up. **9, 10.** Egg chambers, opened for inspection. **11.** Close-up of anterior end of egg showing difference between relatively smooth chorion at apex and reticulated chorion below. **12.** Outer covering of brood chambers as larvae start cocoon spinning. **13.** Same chambers as in figure 12, but now with one opened for inspection. **14.** Same chambers, but now with more outer covering removed to reveal separation of specimens from one another. **15.** Chambers from another nest with some larvae removed to shows start of cocoons that separate larvae. **16.** Larva removed from chamber and kept alive while producing silk, demonstrating texture and color of cocoon silk. **17.** Lower part of two cells from which adult bees had emerged, inner view, demonstrating dark nature of deposited feces and caste white exuviae of pupae (arrows).

to bending of the chorion (Rozen, et al., 2018: figs. 18–22). With *B. impatiens,* only the corners of the hexagons raise to the maximum height above the thin basal chorion, at which point they expand into the mushroom-shaped apices while the sides of the hexagons remain low and therefore not as effective in bracing against bending of the entire chorion as in *B. griseocollis*. However, if the entire chorion bends to the extent that the swollen "mushroom heads" meet, they then provide effective resistance. This system in *B. impatiens* allows more flexibility for moderate or low chorionic bending, but provides a safeguard against excessive bending.

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Worker Eggs: C.S.S. separated two adult workers that emerged from cocoons of Nest 2 and placed them in a separate container to watch their activities. They made a small nest of wax containing two nectar pots as well as a waxen foundation. On 30 January 2018, after the two workers were temporarily removed from the container, a small oblong chamber constructed on the foundation was carefully opened with probe and forceps to reveal five curved feeding young larvae (instars 1 or 2), obviously the offspring of one or both adult workers and hence male larvae since the colony contained no adult males. After the two workers were returned to the nest, they resealed the chamber containing the young larvae within several hours.

Eggs produced by workers were also collected from Nest 3 (listed below under Material Studied). They were obviously unfertilized and therefore would have developed into males. Although not differing in form and chorionic ornamentation from those produced by a queen, their measurements indicate that they tended to be somewhat smaller.

LARVAL INSTARS OF *BOMBUS IMPATIENS*

Bombus impatiens develops through four larval instars, as indicated above. The instars are similar in appearance though obviously varying in size. Because last larval instars of bees are customarily described, a formal description of the fourth (last) larval instar is presented first, accompanied by illustrations of important anatomical features. Then the three earlier instars are diagramed in lateral view, and the features by which they vary from one another and from the fourth instar are explained.

Last (Fourth) Larval Instar

Figures 53–57, 61

Diagnosis: A detailed survey of *Bombus* larvae may eventually reveal distinct differences between species and subgenera on the basis of larval stages. However, with the current paucity of both specimens and literature accounts such a study is not possible, and, furthermore, larvae are morphologically similar. The last stage larva of *B. impatiens* tends to have less integumental pigmentation on its head, and its spiracles and basal labral sclerite are less pigmented than those of *B.* (*Pyrobombus*) *bimaculatus* Cresson, *B.* (*Fervidobombus*) *pennsylvanicus* (De Geer), *B. (Fervidobombus) steindachneri* Handlirsch, and probably *B. griseocollis* (all new information). However, *B.* (*Pyrobombus*) *vosnesenskii* Radoszkowski appears to have even less dark pigmentation on its labrum and maxillae. In terms of head structure, available larvae appear nearly identical. A notable unifying feature of many species is the strongly expressed, internal epistomal ridge connecting the anterior tentorial pits across of the front of the head and its connection with the median longitudinal head ridge, with one apparent exception in that Grandi (1961: fig. 401) illustrated the head capsule of the larva of *B.*(*Thoracobombus*) *sylvarum* (L.) with these structures missing, although he certainly identified them in *B.* (*Thoracobombus*) *pascuorum* (Scopoli) (Grandi, 1961: fig. 406-1).

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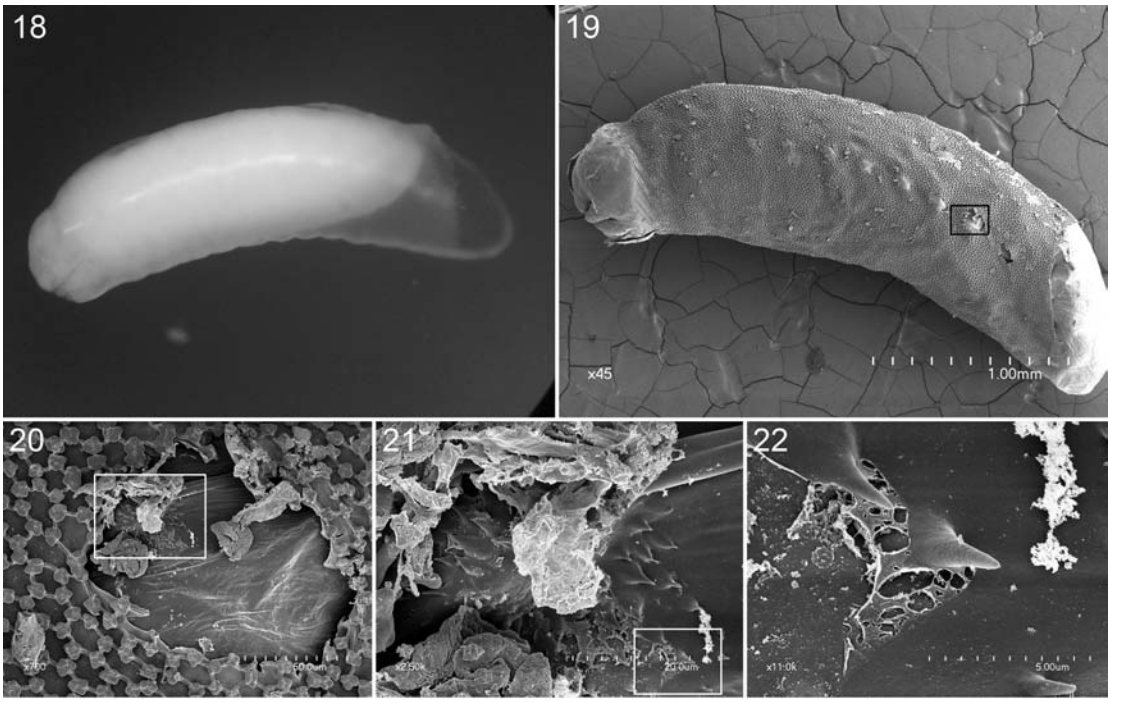

FIGURES 18–22. Preserved pharate first instar of *Bombus impatiens* still completely covered by fully expanded chorion. **18.** Microphotograph of specimen showing position of shrunken first instar in relation to chorion. **19.** SEM micrographs of same specimen of pharate first instar except with slight damage to head resulting from critical point drying. Chorion now revealing early evidence of eclosion in swellings on chorion over where abdominal spiracles had been before shrinking; position of abdominal spiracle 7 (identified with rectangle) on chorion but serosa covered hatching spines and spiracle visible possibly are spiracle 8 brought forward by shrinkage of larva. **20.** Close-up of rectangle in figure 19 around opening of chorion. **21.** Close-up of rectangle in figure 20 showing rosette of spines surrounding spiracular opening hidden by pale amorphous debris. **22.** Close-up of rectangle in figure 21 showing spines appearing to destroy serosa.

Description: **Head** (figs. 54, 55): Sclerotized integument of head capsule faintly pigmented except articulating points of mandibles with hypostomal ridge darkly pigmented; clypeus and frons without patterning of dark marks.

Labrum ringed by basal narrow band of moderately dark pigmentation on dorsal surface and sides; darkening of apical edge resulting from transverse narrow apical band of dark pigmentation showing through from adoral surface; on cleared specimen this band curving posteriorly to base of labrum on each side where it ends. Apex of maxilla faintly pigmented; articulating arm of stipes strongly pigmented; maxillary palpus tending to be darkly pigmented. Prelabium bearing strongly pigmented, subapical, sclerotic ring sublaterally including narrow dorsal bridge; pigmentation of ring gradually diminishing toward venter; extreme labial apex unpigmented except for palpi and salivary lips; apex of labium bearing small, distinct dark mark medially, located internally on salivary duct behind salivary lips. Integument of head capsule and mouthparts with scattered moderately short setiform sensilla.

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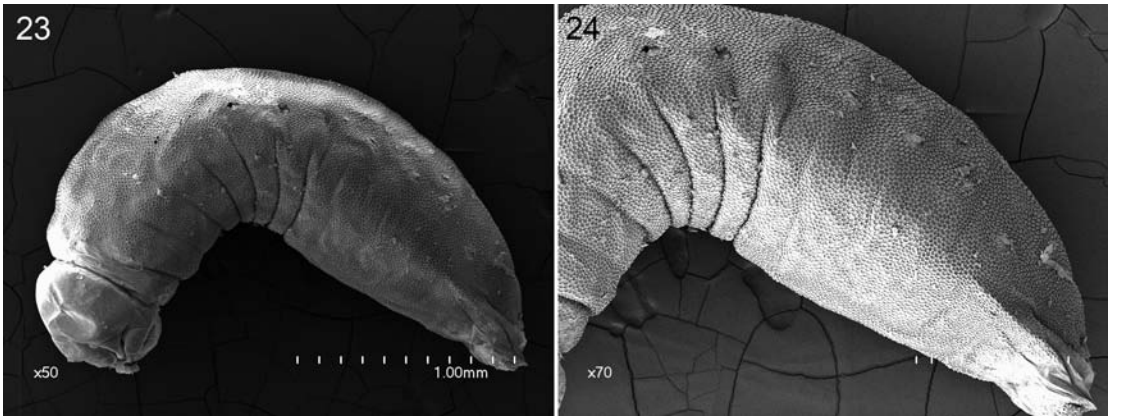

FIGURES 23, 24. SEM micrographs of first instar of *Bombus impatiens* that is starting to emerge from chorion with head now uncovered. **23.** Entire first instar. **24.** Close-up of rear part of body, revealing chorionic apertures over abdominal spiracles and collapsing of chorion at posterior end.

Head size small relative to body size of large individuals (figs. 53); front of head in lateral profile (fig. 55) relatively flat below narrow vertex, so that frons, clypeus, and labrum closely aligned and angling sharply from jutting labiomaxillary region; head capsule moderately broad with summit of vertex more strongly bilobed in frontal view (fig. 54) than that of *B. griseocollis* (Rozen et al., 2018: fig. 27). Tentorium incomplete because of impending pupation; posterior tentorial pits normal in position; posterior thickening of head capsule moderately well developed, only slightly bending forward medially as seen in dorsal view; coronal ridge pronounced internally but unpigmented on cleared head capsule, extending to intersection of pronounced epistomal ridge (fig. 54); these ridges similar though not identical to those of *B. griseocollis* (Rozen et al. 2018: fig. 27). Parietal bands long but not well defined. Antennal prominences scarcely developed (fig. 55); antennal papilla small, but projecting, and longer than basal diameter, bearing perhaps three sensilla. Vertex narrowly rounded in lateral view; frontoclypeal area not projecting beyond labrum (fig. 55); labrum vaguely bilobed.

Mandible (figs. 56, 57) apically darkly pigmented, nearly black; gradually becoming lighter toward base except for two dark areas, i.e., one at each articulation with head capsule; mandibular shape (figs. 56, 57) stout, rather short, with long axis slightly curving upward in inner (fig. 57) and outer views; mandibular apex rounded with pronounced scoop-shaped concavity (fig. 57) on inner surface occupying approximately two-fifths of mandibular length; dorsal edge of concavity bearing single subapical tooth partway to base; this tooth slightly splayed outward apically; edge of concavity moderately smooth; outer surface of mandible somewhat uneven midlength, bearing single fine seta at base.

Labiomaxillary region produced and not greatly fused; labium projecting somewhat beyond maxilla in lateral view (fig. 55). Maxillary apex not bent mesad; palpus apical in position, elongate, more than twice as long as basal diameter, pigmented; galea represented by pair of stout but short setae arising from small tubercle on inner edge of maxillary apex short distance mesad from palpus; stipes apically partly ringed by darkish sclerotization except extreme apex unpigmented; articulating arm of stipes pronounced, darkly pigmented; basal articulation of stipes to cardo faintly pigmented;

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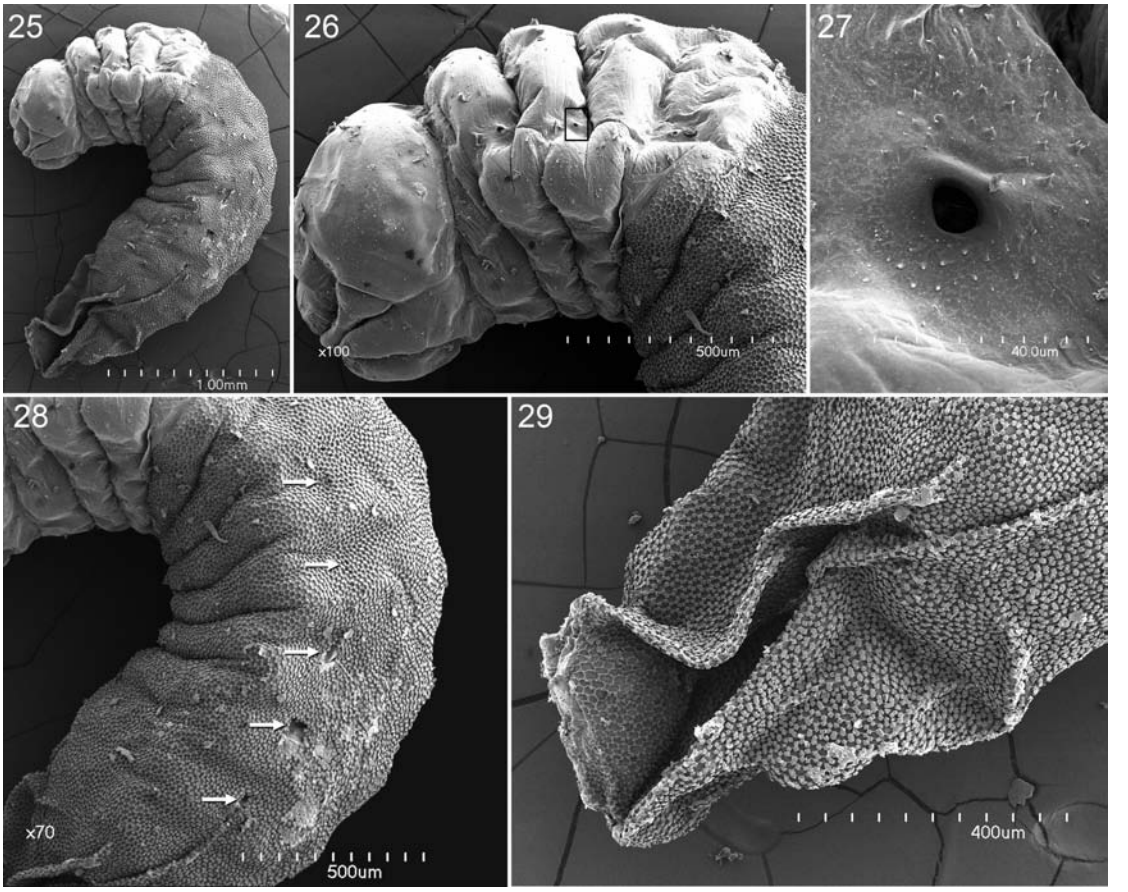

FIGURES 25–29. SEM micrographs of first instar of *Bombus impatiens* with head and thoracic segments now free of chorion. **25.** Entire larva, lateral view. **26.** Close-up of front of body showing position of uncovered spiracles with second thoracic spiracle identified (rectangle). **27.** Close-up of second thoracic spiracle showing rosette of spines. **28.** Close-up of rear end of body showing series of apertures (arrows) that had opened over spiracles when hatching process started. **29.** Close-up of caste chorion at posterior end of larva.

cardo more or less pigmented. Strongly projecting labium divided into prementum and postmentum, and bearing apically projecting broad lips of slitlike salivary opening, its transverse length as long as distance between labial palpi; labial palpus with length more than 2× basal diameter. Hypopharynx single pronounced mound, strongly spiculate at base but not at summit.

Body: Integument generally densely covered with extremely fine, minute, sharp-pointed spicules except for inter- and intrasegmental creases; on midbody segments (approximately abdominal segments 3 and 4) spicules on outer surface of lateral abdominal swellings gradually becoming somewhat less dense in contrast with those of *B. griseocollis*; on midbody segments, vestiture of caudal annulets consisting of spicules and wide scattering of fine setae, more than 4× longer than spicules; these setae also present and moderately abundant on abdominal segment 10 below slit-like anus. Thoracic segments each with pair of small, pointed, apically sclerotized and pigmented tubercles dorsally (fig. 53) as characteristic of corbiculate taxa, but

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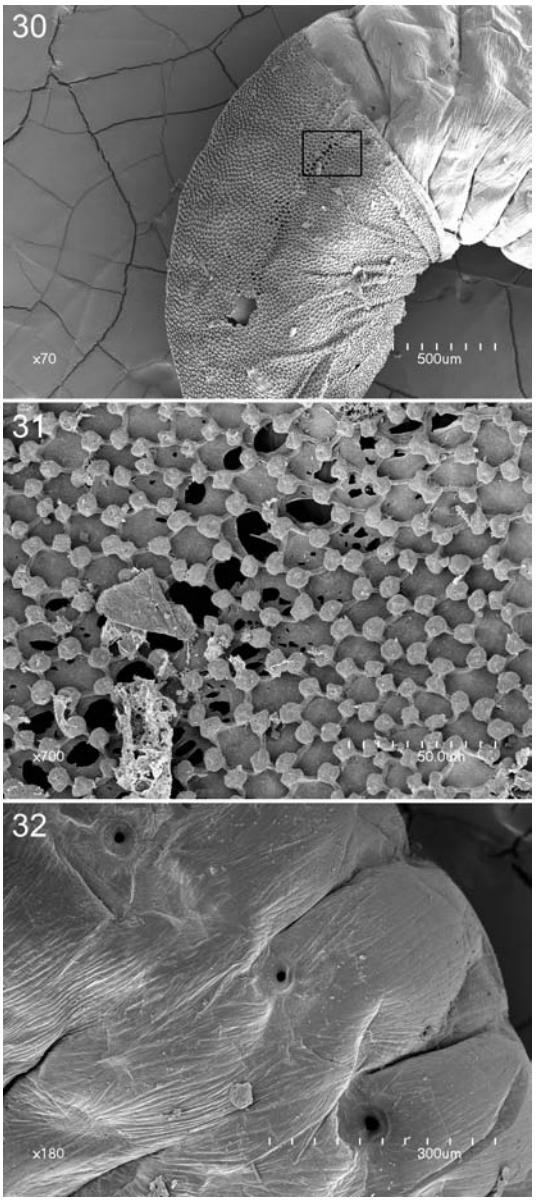

FIGURES 30–32. SEM micrographs of another hatching first instar of *Bombus impatiens* about halfway out of chorion, anterior end to right. **30.** Rear of body showing disruption of chorion along spiracular line. **31.** Close-up of destruction of inner layer of chorion along spiracular line identified by rectangle in figure 30. **32.** Close-up of thorax and first abdominal segment demonstrating enlarged first thoracic spiracle compared with two following spiracles.

these tubercles not found on any abdominal segments; spiracles exhibiting unpigmented atrial rims and spines (see below), elsewhere integument unpigmented. Modification of larval shape during development of pupa not observed, but likely as described for *B. griseocollis*. Spiracle (fig. 61) of defecating larva with rim unpigmented, slightly projecting beyond body wall, peritreme moderately narrow, composed of thin cuticle often difficult to identify; atrium shallow, less than half as deep as wide; atrial wall ringed with parallel ridges bearing dense mass of long, concentrically directed unpigmented spines; primary tracheal opening with or without spines depending on interpretation of spines encircling opening between atrium and subatrium; subatrium short ring, slightly wider than base of atrium as viewed externally (fig. 61), consisting of only one to three indistinct narrow sclerotized rings; flexure a thin flexible cuticular tube without spiraled taenidia extending between subatrium and tracheal system identified by taenidia; flexure, usually longer that atrium plus subatrium, enabling separation of outer surface of larval from massive internal mass of larva in connection with locomotion (Rozen, 2017).

Material Studied: Three postdefecating larvae, from nest purchased 2–3 weeks earlier from Biobest, collected 29 Nov. 2017 at Princeton University, by all of us. Thirty-two post- and predefecating last-stage larvae, from nest purchased from Biobest, collected 11 January 2018 at the American Museum of Natural History by J.G.R. and C.S.S.

Earlier Larval Instars

Figures 50–52, 58–60

As is characteristic of bees in general, larvae of *B. impatiens* grow remarkably in size

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after hatching from eggs that are less than 4 mm long and about 1 mm in maximum diameter. Except for size, the first three larval instars are quite similar as seen in lateral view (figs. 50–52) in that they tend to be strongly curved and have linear bodies with body segmentation only weakly obvious, as is also true of the cephalic-caudal annulation of abdominal segments. With continuous ingestion of food, the posterior part of the body starts to become physogastric during the second instar as is evident by the third instar (fig. 52). In that instar, small, paired dorsal thoracic tubercles are elevated though neither as evident nor as darkly pigmented as those of the fourth instar. Paired dorsal thoracic tubercles of the last larval instar appear to be characteristic of all known corbiculate bees. In the Meliponini (Michener, 2007), they are reported to also occur on some of the abdominal segments. Because they appear at their maximum elevation and pigmentation only in the last larval instar, this suggests that they fulfill some unknown function characteristic of that stage. Typically, the last larval instar of all bees must position itself to deposit feces in a limited part of the brood chamber. Further, cocoon-spinning larvae must move around in the chambers to deposit silk to all surfaces of the chamber wall. Hence, future observations may indicate that these tubercles assist the larva by providing traction, leaving unexplained how other larvae deposit feces in restricted parts of brood chambers and spin cocoons without such tubercles.

The head capsule of the first instar of *B. impatiens* is weakly but distinctly sclerotized. Mandibles are fully formed and already scoop shaped. Major parts of the maxillae and labium are distinctly if weakly sclerotized, but palpi are scarcely longer than their basal diameters. The body is extensively covered with minute spicules. An interesting unexplained phenomenon was noted on the dorsal surface of the first instar still covered by the accidentally broken chorion in that the dorsal spicules bore elongate multiple, fingerlike extensions (fig. 40), possibly created by the serosa. Such phenomena were not seen on first instars that had naturally emerged from their chorions.

In the second and third instar sclerotic areas of the head become more pronounced, palpi more elongated, and body spicules continue to become more apparent while body setae gradually lengthen though remaining sparse. On cleared specimens, spiracles (figs. 58–61) always have pronounced atrial spines concentrically directed. The atrium of the first instar is shallow compared with its width (fig. 58) but becomes deeper in subsequent instars, while the subatrium remains apparent. The flexure is surprisingly long and slender in the first instars but remains quite transparent and is always seen connecting to the tracheal system identified by taenidia. Occasionally specimens of instars are preserved as they are nearing molting to the next larval instar, at which time the atrium and trachea of the next instar appear, surrounding the atrium and trachea of the current instar (fig. 62).

In final analysis, the changes that take place from one larval instar to the next in this species are seen to be gradual.

Material Studied: Nest 1: First instar: 17 specimens; second instar: 11 specimens; third instar: 12 specimens. Nest 2: First instar: 17 specimens; second instar: 24 specimens; third instar: 23 specimens.

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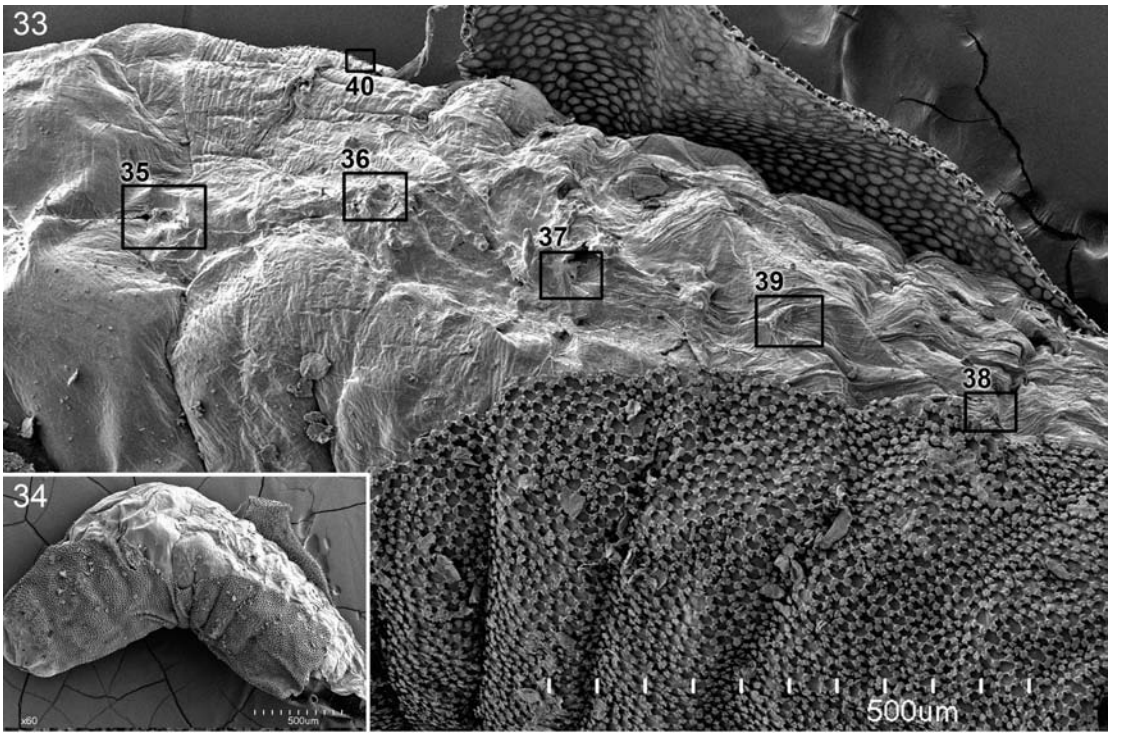

FIGURES 33, 34. SEM micrographs of egg with chorion broken exposing serosa covered first instar, anterior end to left. **33.** Close up of most of abdomen of first instar exposed, showing spacing of 5 of the 8 spiracles identified along one side of body, assuming first spiracle identified is abdominal spiracle 2. **34.** Entire egg with fractured chorion partly attached.

PUPA OF *BOMBUS IMPATIENS*

The pupa of *B. impatiens* corresponds in all respects with that of *B. pennsylvanicus* De Geer) (as *B. americanorum* (Fabricius)) and *B. vosnesenskii* Radoszkowski based on characters identified by Michener (1954). That is to say, there are no pupal features of the head or mesosoma except for the legs. The following description refers to the pupal features shared by these three species. Specimens of only female pupae of *B. impatiens* were available.

Description: All coxae with inner apical spine; all trochanters with posterior apical spine. Metasomal terga 2–5 each with apical row of fine long spicules.

Material Studied: 7 pupae from nest purchased 2–3 weeks earlier from Biobest, collected 29 Nov. 2017 at Princeton University, by all of us. Fifteen pupae from nest purchased from Biobest, collected 11 January 2018 at the American Museum of Natural History by two of us J.G.R. and C.S.S.

DISCUSSION AND CONCLUSIONS

From this study the nesting biology of *Bombus impatiens* resembles that summarized and described by Michener (2007: 788–790) for the nonparasitic bumble bees in that queens deposit

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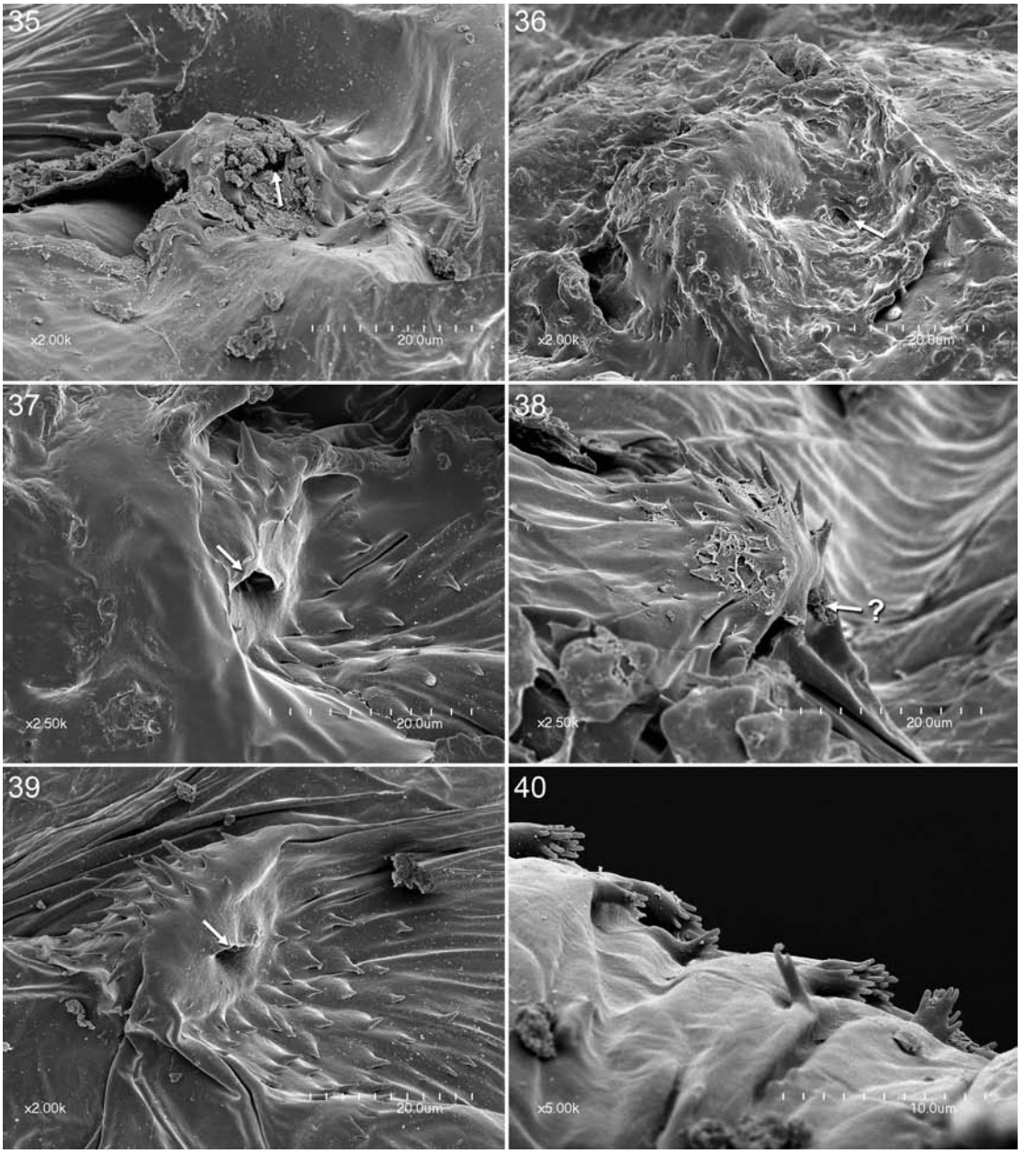

FIGURES 35–40. Same specimen as figures 33 and 34 (with spiracular opening identified by arrows). 35. Centered, presumed abdominal spiracle 2. 36. Presumed abdominal spiracle 3. **37.** Presumed abdominal spiracle 4. **38.** Presumed abdominal spiracle 5. **39.** Presumed abdominal spiracle 6. **40.** Close-up of dorsal surface identified on figure 33 by bracket showing spicules bearing elongate, multiple, fingerlike extensions.

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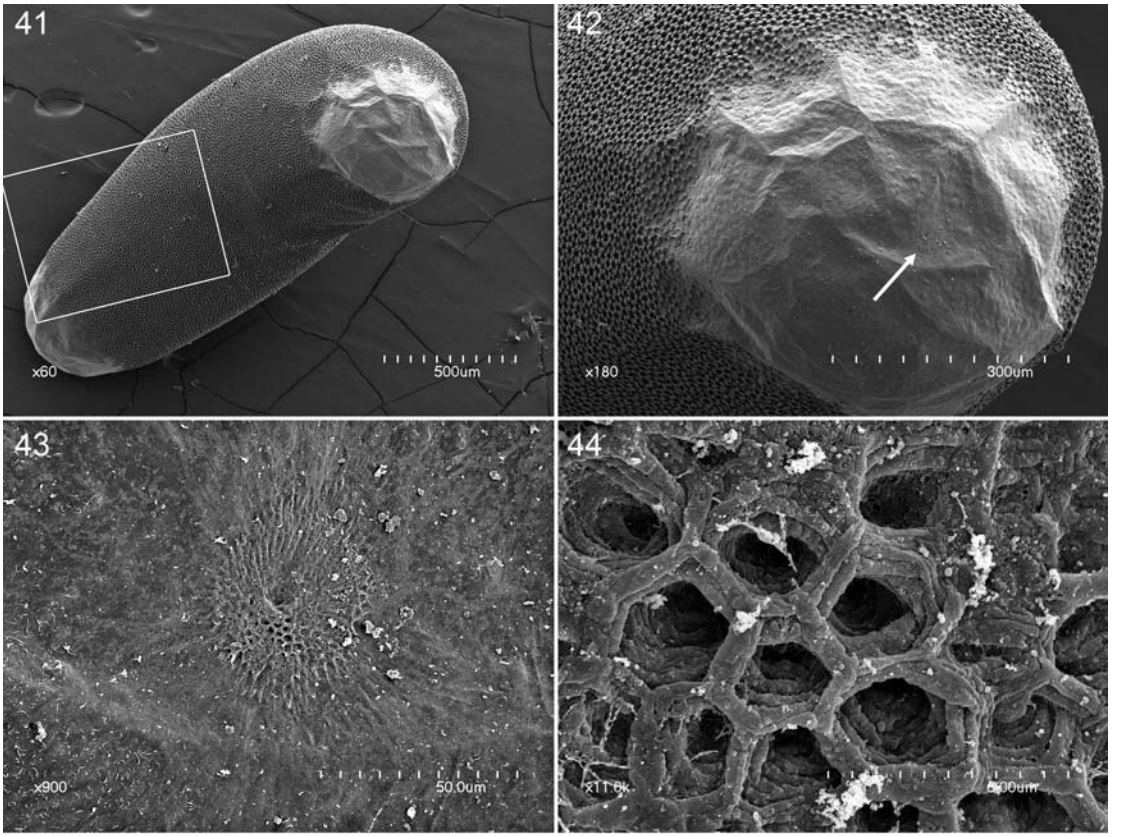

FIGURES 41–44. SEM micrographs of egg of *Bombus impatiens*. **41.** Entire slightly curved egg anterior end at right showing pale, nearly smooth chorion at front and rear ends and darker, densely reticulate chorion between two ends. **42.** Close-up of front end showing position of micropyle (arrow). **43.** Close-up of entire micropyle. **44.** Further close-up showing complex structuring of micropylar pores.

numerous eggs into a single chamber, and larvae, as they feed and grow, divide and re-form the chamber, so that each last larval instar is separated in its own cell.

Much of the information on eggs and larvae of this species is new because of revelations provided by SEM examination and almost certainly will invite comparative studies of other species of nonparasitic *Bombus.* Examination of parasitic forms is even more likely to produce novel insights in larval development and anatomy since these forms have traditionally been given less priority.

As reported above, hatching spines arranged as rows just above the spiracular line on the two sides of hatching larvae have not been detected in the case of *B. impatiens.* With that species, spines associated with spiracles on first instars form a small group around each spiracle, presumably resulting only in the tearing of the chorion immediately exterior to it, unlike the situation with most bees, as presented in the section entitled Egg Eclosion. We do not understand why other bees need to tear the chorion along both sides of the egg in order to emerge whereas *B. impatiens* sheds the chorion without its splitting along the sides. Further studies of *B. impatiens*

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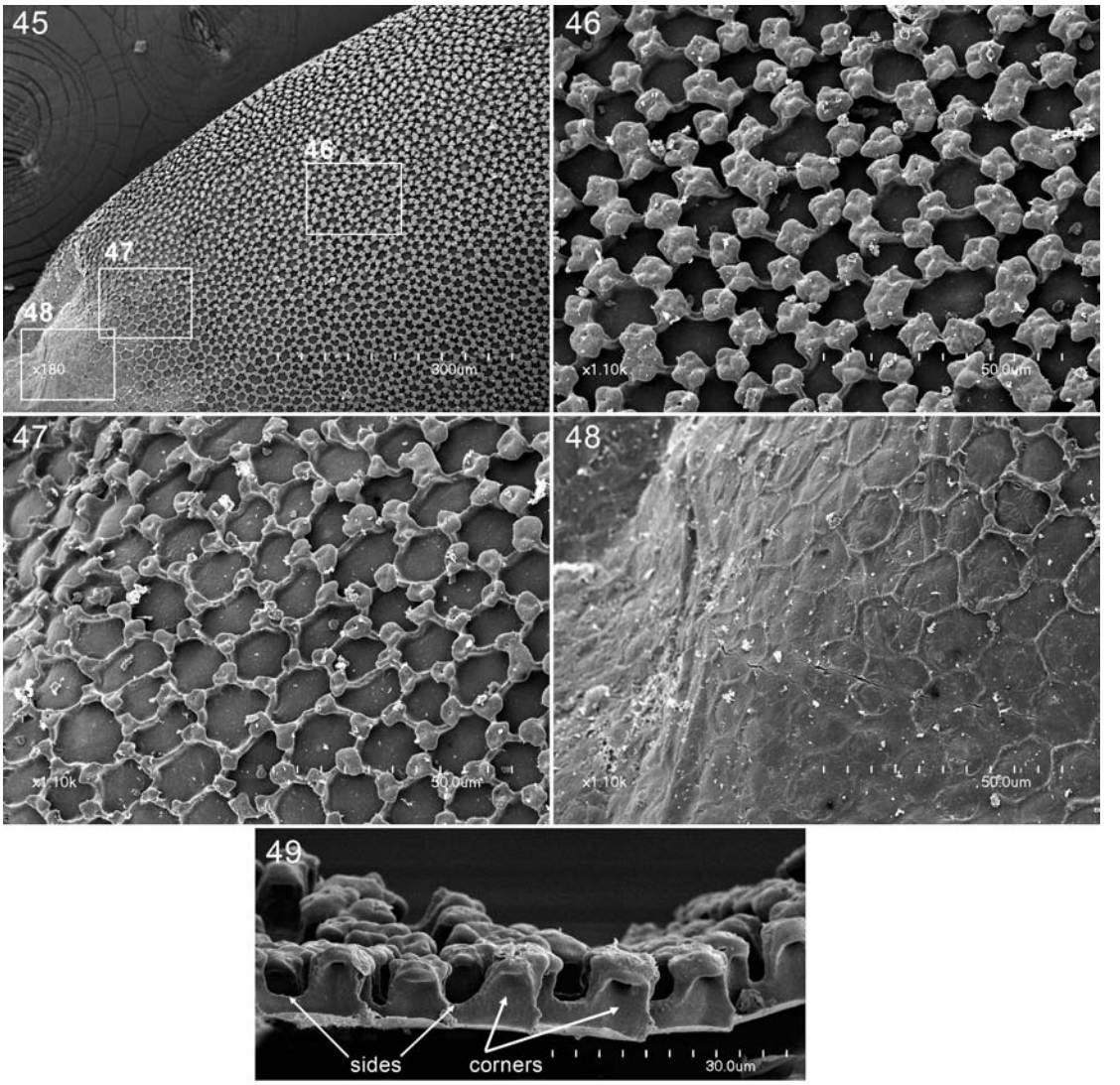

FIGURES 45–49. SEM micrographs of structure of reticulations on figure 41. **45.** Close-up of densely reticulate chorion (rectangle in fig. 41) showing diminution of elevations of reticulation approaching posterior end. Following three figures showing close-ups of extent of expression of hexagons on surface: **46.** Reticulations at maximum elevation. **47.** Reticulations with elevations decreasing toward posterior end of egg. **48.** Reticulations nearly disappearing at posterior end of egg. **49.** Close-up of broken edge of chorion showing corners of hexagons extending upward above thin base of chorion and expanding into mushroomlike protuberances well above sides of hexagons.

should probe these observations and study them further. Investigations of additional species will be needed to determine whether other species of *Bombus* exhibit the same phenomena.

As another thought provoked by this study, this species exhibits only four larval instars whereas most bees pass through five such stadia. Is it reasonable to ask: Which instar has been

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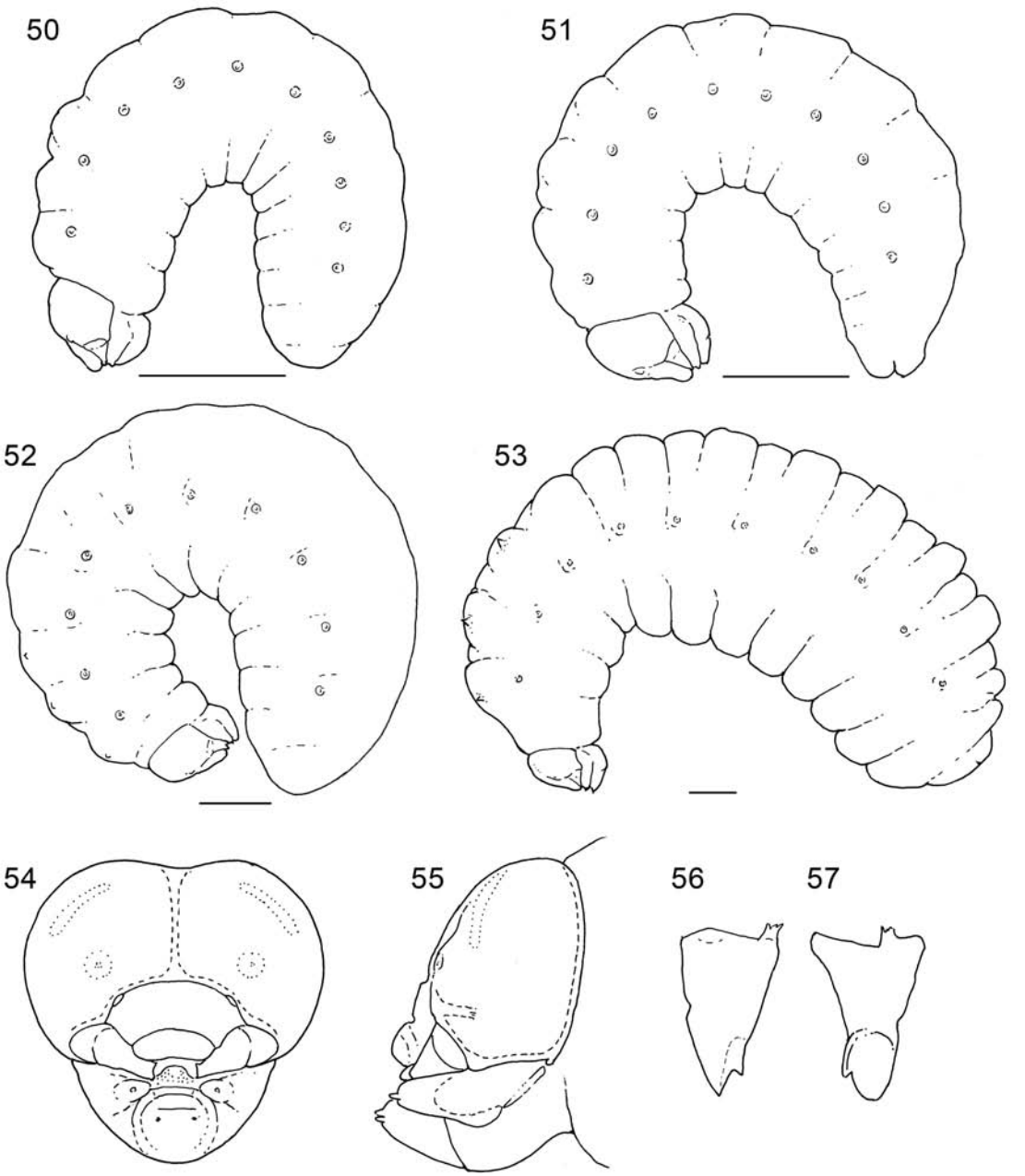

FIGURES 50–57. Diagrams of larval instars of *Bombus impatiens*; scale bars = 1.0 mm. **50.** First instar, lateral view. 51. Second instar, lateral view. **52.** Third instar, lateral view. **53.** Fourth instar, lateral view. **54, 55.** Head of fourth instar, frontal and lateral views, respectively. **56, 57.** Right mandible of fourth instar, dorsal and inner views, respectively.

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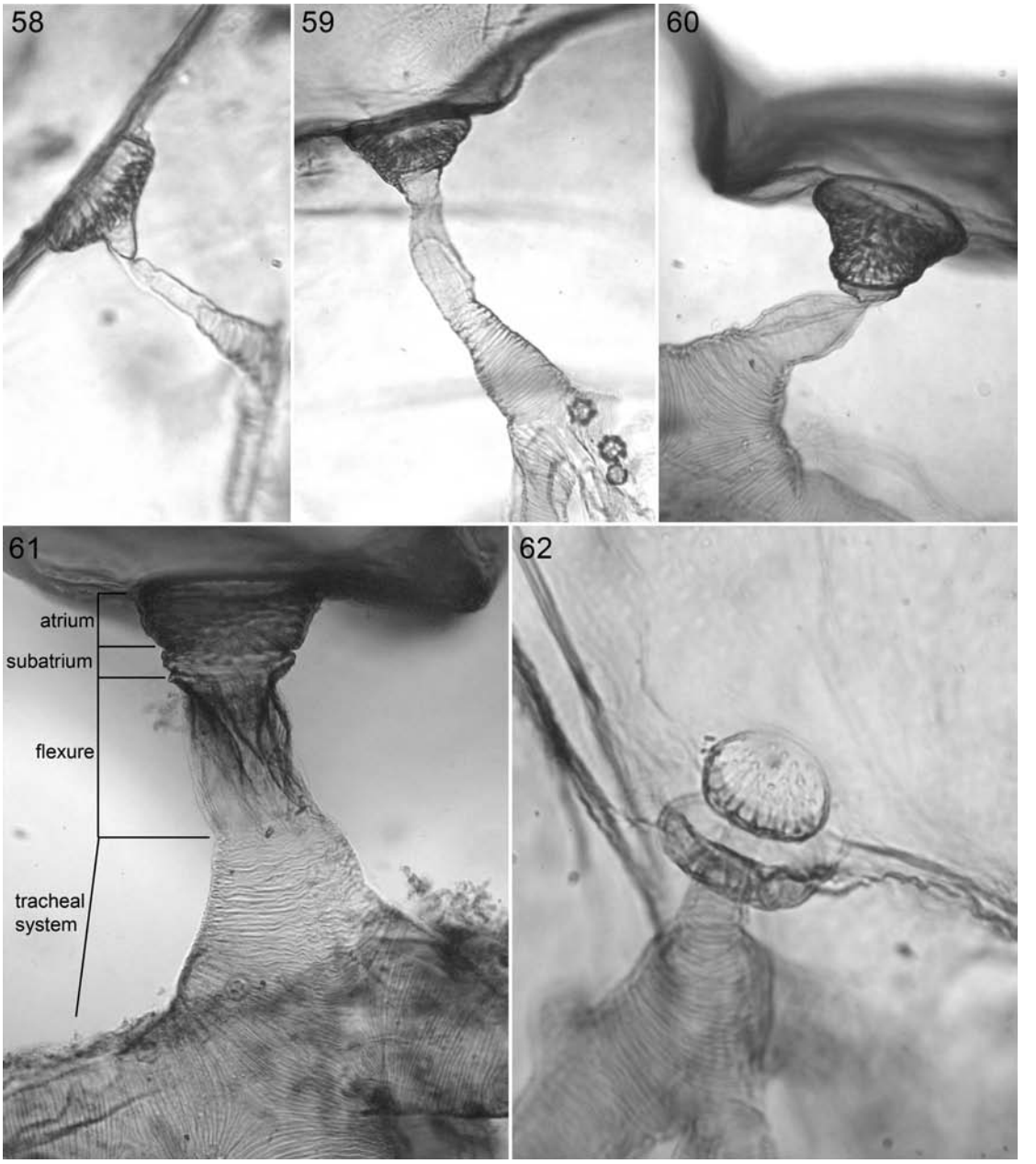

FIGURES 58–62. Microphotographs of spiracles of cleared larval instars of *Bombus impatiens*, mostly side views. **58.** Spiracle of first instar, showing very shallow atrium, narrow subatrium, long slender flexure connecting to branching tracheal system identified by taenidia. **59.** Second instar, as in first instar, but now atrium becoming more bell shaped. **60.** Third instar, as in previous instar, but atrium increasing in size; flexure and connection to tracheal system relatively short compared to those of previous instars. **61.** Spiracle of fourth instar. **62.** Spiracle of fourth larval instar developing around base of third instar.

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lost? If the first, might the method of eclosion exhibited by *B. impatiens* be a substitute for the absence of a linear row of spines along the spiracular line of true first instars?

In addition to future studies of Euglossini and Bombini, nesting biology of the last group of corbiculate bees, the Meliponini, is underway with the gathering of specimens of representative taxa.

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