Did developing brood drive the evolution of an obligate symbiosis between ants and bacteria?

Serafino Teseo^{1†}

¹School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive Singapore 637551

†To whom correspondence should be addressed: steseo@ntu.edu.sg

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Abstract

Blochmannia is a vertically transmitted obligate bacterial symbiont of ants within the tribe Camponotini (Formicidae: Formicinae), hosted in specialized cells (bacteriocytes) of the ant midgut epithelium. Genomic comparisons of *Blochmannia* with other insect symbionts suggest that the symbiosis may have started with ants tending sap-feeding insects. However, the possible transitions of *Blochmannia* from mutualist of sap-feeding insects to vertically transmitted organelle-like symbiont of ants have not been formally discussed. Here I propose hypotheses supporting the idea that the ant brood may have had a prominent role in this process. This is mainly because: 1) microbes are more likely to reach the midgut in larvae rather than in adults; 2) bacteriocytes possibly allowed the midgut lumen-dwelling ancestor of *Blochmannia* to survive gut purging at the onset of the ant pupation, extending its nutritional benefits to metamorphosis; 3) adult ants do not need the nutritional benefits of *Blochmannia*. Investigating the biology of Camponotini sister taxa may provide further cues regarding the evolution of the symbiosis.

1. Introduction

Obligatory symbioses involving intracellular maternally transmitted microorganisms with simplified genomes (primary endosymbioses) are common across insects [1]. These are thought to begin with the establishment of free-living bacteria that provide nutritional benefits to the host and gain a stable environment to live in [2]. Following this, the insect host increasingly relies on the bacterial physiology and the microorganism becomes more and more dependent on it, undergoing genomic shrinkage and simplification [3]. Finally, symbiotic bacteria and the insect reproductive system coevolve and the microbe gains access to the host germline, becoming vertically transmitted. In most cases, symbiont vertical transmission occurs from soma to germline (hereafter, soma-to-germline vertical transmission, or SGVT) [2].

The γ -proteobacterium *Blochmannia* is an obligatory intracellular symbiont of ants from the tribe Camponotini (Formicidae: Formicinae) [4,5], exclusively transmitted vertically from mother to offspring (queen to worker) and never horizontally, e.g., via mouth to mouth food exchanges [5]. *Blochmannia* enters developing oocytes via the follicle cells [6] in the ovaries of queens. It is then hosted in specialized cells (bacteriocytes) within the midgut epithelium in developing larvae/pupae and young adults [7]. *Blochmannia*'s most closely related organisms are some secondary bacterial endosymbionts of sap-feeding mealybugs (Hemiptera: Pseudococcidae) [8]. Sap-feeding insects often associate with ants (including Camponotini), providing them with sap sugars and earning protection benefits from them. As all Camponotini ant genera examined so far (*Calomyrmex*, *Camponotus*, *Colobopsis*, *Echinopla*, *Opisthopsis*, *Polyrhachis*) [8–10] are in symbiosis with *Blochmannia*, it is thought that the *Blochmannia* Ancestor (hereafter: BA) was originally acquired by the common Camponotini Ancestor (hereafter: CA) via tending sap-feeding insects [8].

The 700kb genome of *Blochmannia* encodes for urease and glutamine synthetase, making it able to recycle nitrogen into amino acid biosynthesis [11,12]. *Blochmannia* therefore enhances the ant host nutrition [12] through the activity of these genes, which is considered a key feature of the symbiosis. This nutritional upgrading is correlatively supported by experimental evidence that antibiotic-treated

individuals and colonies are less viable than controls [12,13], and that antibiotic-treated larvae develop into hypomelanistic adults with a thinner cuticle [14].

Blochmannia levels are highest across ant metamorphosis and early adult life [15,16]. During metamorphosis, *Blochmannia*-harboring cells within the midgut epithelium form a structure reminiscent of a true bacteriome [17], an organ hosting symbiotic bacteria during development in other insects [18]. This suggests *Blochmannia* optimizes the use of nutrients accumulated during larval stages, which are in high demand during processes occurring during metamorphosis (e.g., cuticle formation [15,14]). As young ants usually take care of feeding developing brood, high *Blochmannia* titers in early adult life are also thought to increase nutrient availability for larvae [19].

2. Hypothetical transitions leading to SGVT establishment

In highly eusocial ants such as Camponotini, queens are virtually the only reproducing individuals. Therefore, SGVT establishment must occur in queens for BA to start passing vertically from one generation to another. The evolutionary steps leading to this, however, have not been discussed. Here I propose a series of hypothetical transitions: 1) CA establishes an association with sap-feeding insects, in which workers and larvae come into contact with their microbial symbionts (BA) via their honeydew and/or by consuming them; 2) a period of facultative symbiosis follows. BA is hosted in the CA gut lumen and upgrades its nutrition; 3) midgut bacteriocytes appear, maximizing the benefits for both symbiotic partners; 4) BA reaches the queen ovaries and SGVT begins.

Two of these events are of paramount importance. First, the evolution of midgut bacteriocytes, which marks the transition of BA from extracellular to intracellular; then, the appearance of SGVT in queens. Bacteriocytes are necessary for SGVT for two reasons: 1) it is not likely that BA started as an exclusive CA germline associate with no selective advantages for the ant host. This would make the evolution of the Camponotini-*Blochmannia* mutualism improbable; 2) as shown for *C. floridanus*, the presence of *Blochmannia* requires a lowered immune response from the ant [20,21]. It is therefore

more likely that BA reached the germline in ants where it was already established in the intracellular environment, rather than in individuals where the immune system precluded it from entering cells.

3. From crop to midgut to bacteriocytes

Bacteriocyte location depends on where the symbiont action is needed [2] and bacteriocytes of *Camponotini* occur in the midgut internal epithelium. It is therefore reasonable to assume that BA dwelled in the ant midgut lumen during the transition phase in which it was a facultative CA endosymbiont. This begs an important question: how did BA reach the ant midgut?

Morphological analyses of the proventriculus (a valve-like structure between crop and midgut) in *Camponotus* workers [22] reveal the presence of a structure retaining extremely small particles [23]. Experimental evidence shows that particles larger than 1µm diameter do not pass from crop to midgut in *Camponotus floridanus* [24]. A fine filtering activity may therefore occur in *Camponotus* and other *Camponotini* ants [23]. Similarly, in distantly *Camponotini*-related *Cephalotes* workers, the proventriculus acts as a “bacterial filter” preventing harmful bacterial strains from disrupting microorganismal communities in the midgut [25]. Assuming that the CA had a bacterial filtering apparatus, and given that *Blochmannia* cells are at least 1µm diameter [7], it is unlikely that the BA passed the CA proventriculus to reach the midgut.

Intriguingly, in most ants and including *Camponotini*, larvae do not have a proventricular filter [22] and are able to ingest relatively large particles. Assuming that CA workers and queens had a proventricular filter and larvae did not, it is more likely that BA first reached the midgut in larvae rather than in adults. This scenario, already proposed in a previous study [8], suggests that bacteriocytes initially appeared in developing brood rather than in adult workers.

What were the selective pressures leading to bacteriocyte emergence in developing individuals? In holometabolous insects, growth only occurs across larval stages, when individuals accumulate nutrients prior to metamorphosis. Facultative gut bacterial symbionts may upgrade nutrition in these stages, maximizing growth, which would justify the appearance of the BA symbiosis. However, there is no

nutrient inflow during costly metamorphosis, and a symbiont-mediated nutrient optimization in this phase could make a significant improvement. Nonetheless, as metamorphosis approaches, gut contents and residing bacteria are purged [26]. A way to extend a symbiont's nutritional upgrading to metamorphosis is by avoiding such a symbiont purging, which may be the reason why BA passed from the gut lumen to inside cells of the midgut internal epithelium.

4. Adult ants do not need the nutritional upgrade of *Blochmannia*

As the Camponotini-*Blochmannia* symbiosis originated around 40 MYA [9], it is not possible to assess whether the proventriculus acted as a bacterial filter in CA. In addition, adult ants from the tribe Camponotini host bacteria in the gut lumen [27,23], implying that these pass the proventriculus. Therefore, other possibilities regarding bacteriocyte evolution and BA SGVT establishment must be considered. One way to do so is by relaxing the assumption that BA could only reach the midgut in developing larvae, and hypothesize that bacteriocytes originated in the midgut of adults. A first argument against this is that contemporary *Blochmannia* is rather a developmental partner than a life-long symbiont [15,17], which makes it more linked to larval/pupal stages than to adult life. Secondly, novel organs such as bacteriocytes are more likely to appear during development rather than after eclosion, especially in holometabolous insects that hardly change after metamorphosis. Finally, and most importantly, adult ants do not need the nutritional upgrade of *Blochmannia*. In adult workers of *Camponotus* ants, *Blochmannia* levels decline with age and bacteriocytes decrease and degenerate [7,15,16], indicating that the symbiont progressively loses importance. Based on these arguments, the emergence of bacteriocytes in adult workers appears to be improbable.

Concerning queens, in established colonies these do not need the *Blochmannia* nutritional upgrading either, as their titers appear not to be higher than those of workers in *Camponotus* and *Colobopsis* [7,10]. This suggests that BA was similarly not needed in established CA queens. On the other hand, BA SGVT may have increased the success of young CA queens during colony founding, when nutrient availability is limited and critical. However, as CA queens were raised in colonies already

hosting BA as a facultative symbiont, they could have carried it in their crop and transmitted it to workers via trophallaxis, without SGVT. Similar SGVT-free dynamics are found in virgin queens of contemporary ants that leave their native colonies carrying symbiotic fungi [28] or sap-feeding insects [29] prior to colony foundation.

5. Alternative scenarios

In the previous sections, I have supported the ideas that bacteriocytes originated in developing brood and that adult CA individuals do not need the nutritional enhancement of *Blochmannia*. But what are the possible scenarios if both of these hypotheses are rejected? Here I explore alternative situations in which bacteriocytes appear in adult individuals and *Blochmannia* is needed by adult ants. In this case, there are two possibilities: 1) bacteriocytes appear first in CA workers and queens acquire them in a second moment, which eventually leads to SGVT establishment; 2) bacteriocytes appear first in queens and then in workers.

Concerning the first scenario, if a facultative symbiotic microbe improves worker traits such as size, lifespan or efficiency in food provisioning, colony production of sexuals may increase because of the increased nutrient availability. As a consequence, symbiont-supporting worker genes may be favored, which would eventually result in colonies increasingly relying on the symbiont. Nonetheless, worker benefits alone do not necessarily increase the chances of SGVT establishment, which instead must occur via the queen. Even though a colony performs optimally due to a facultative bacterial symbiont living in worker bacteriocytes, this does not increase chances for queens to evolve SGVT, which must provide selective fitness advantages in order to appear and invade the population. If this does not occur, a facultative symbiont with benefits for workers can still pass through generations via founding queens transmitting it to their workers via trophallaxis, or be re-acquired from the environment at each generation. Contrarily, SGVT establishment in queens could occur via kin selection if the symbiont invades the ovaries of workers, but only if this provides selective fitness advantages to the colony.

However, given that workers do not reproduce, there are no such advantages, which makes queen SGVT establishment via kin selection a possible but merely stochastic event.

In the second scenario, BA SGVT evolution in queens independently from workers is expected if it increases a queen's fitness. A possibility is that BA SGVT increased colony founding success in CA queens, and in fact, *Blochmannia* is thought provide such an advantage in Camponotini [30]. However, this function may also have evolved in a second moment, when the symbiosis was already established. Indeed, as mentioned above, founding queens could have spread BA in their new colonies via trophallaxis, without needing SGVT.

Lastly, it cannot be excluded that BA SGVT may have emerged because it provided CA queens with selective advantages that were relevant at the time but cannot be identified today as they disappeared throughout evolution. However, according to comparative analyses of *Blochmannia* strains across genera within extant Camponotini, BA's genome only went through moderate changes [9], making this hypothesis less viable.

6. Conclusions

In this study, I formulated hypotheses that tentatively reconstruct the evolutionary steps leading to *Blochmannia* SGVT establishment in Camponotini. The proposed transitions towards SGVT include the following scenarios: 1) workers are in contact with BA-hosting sap-feeding insects (Fig 1a); 2) workers transmit BA to larvae (Fig 1b); 3) BA colonizes larval midgut bacteriocytes that persist through metamorphosis (Fig 1c). *Blochmannia* reaches ovarian follicles, finally leading to inheritable SGVT (Fig 1d). It is not clear how this last transition may have occurred. However, as *Blochmannia* occasionally invades cells other than gut bacteriocytes during metamorphosis [6], a possible explanation is that BA may have invaded other tissues, including precursor cells of ovarian follicles.

The bacteriocyte-ovary transition had to occur in queen-destined larvae for SGVT to appear. It could be even hypothesized that, at early stages in the evolution of the symbiosis, nutritionally upgraded individuals were larger than others at the onset of metamorphosis, and therefore more likely to

differentiate into queens [31]. BA-induced queen determination could have promoted the BA-CA interdependency and consequently the appearance of bacteriocytes.

Other mechanisms may have concurred in the establishment of BA in the CA's midgut. Proteins in *Camponotus* [32,33] and formic acid across the ant subfamily Formicinae [34] act as a chemical filter selecting specific bacterial strains in the gut. Formic acid creates an acidic environment where only few bacterial strains survive and others, including pathogens, cannot establish [34]. This is only documented in adult ants, but since larvae are fed by workers, acidic environments are probably also present in larval guts. As formic acid-mediated gut acidification seems to be a primitive trait of Formicinae, probably also CA workers swallowed their formic acid, and BA must have survived this process in its route towards SGVT. This suggests that BA and *Blochmannia* thrive in acidic environments, but to my knowledge there are no measures of the pH of ant gut bacteriocytes.

Finally, it is still unknown why the *Blochmannia*-Camponotini symbiosis appeared in the first place, and what was the biology of CA before BA acquisition. It can be hypothesized that, prior to BA, CA workers fed larvae with relatively more nutrient food, and that BA allowed to increase the number of larvae raised to adulthood or to colonize ecosystems where nitrogen was less abundant. A way to explore these hypotheses may be investigating the biology of the sister taxa of Camponotini, such as the *Blochmannia*-free ant genus *Notostigma* [8]. For example, determining the presence/absence of vestigial bacteriocytes in *Notostigma*, the composition of its gut microbiota and the basic ecology of the genus may be relevant for reconstructing the evolution of the *Blochmannia*-Camponotini association.

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208 **Figure 1.** The proposed transitions leading to the establishment of soma to germline vertical transmission
 209 (SGVT) in the Camponotini Ancestor (CA). **a.** CA workers tend to sap-feeding insects, coming in contact with
 210 BA. **b.** CA workers pass the BA to larvae by feeding them sap-feeding insects and/or via trophallaxis. **c.**
 211 Appearance of bacteriocytes in larvae allows BA to persist through metamorphosis. **d.** BA reaches the
 212 ovarian follicle cells within developing queens, leading to the establishment of SGVT.

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