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Research article

Allantoin Crystal Formation in *Bagrada hilaris* (Burmeister) (Heteroptera: Pentatomidae) Females

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Abstract

Bagrada hilaris is a polyphagous herbivore reported as an invasive pest in the United States. During the course of dissecting *Burmeister hilaris* unique crystals were observed in both the midgut and oviducts. Crystals were identified using X-ray diffraction techniques. Both acicular (i.e., needle-like, slender, and/or tapered) and cubic (i.e., cube shaped) crystals were observed in six of 75 individuals examined (8.0%). The crystals were mainly observed in females (6.7%), followed by males (1.3%) with no crystals observed in the minimal number of nymphs examined (0%). Crystals of both types were detected in the midgut and lateral oviducts of the females and midgut in males. The acicular crystals often appeared as distinct bundles when present in the midgut and oviducts. Crystals varied in size with the acicular crystals ranging from 0.12 mm to 0.5 mm in length although the cubic crystals ranged in length from 0.25 mm to over 1.0 mm with widths of ~0.25 mm. The cubic crystals were identified as allantoin although the acicular crystals were most likely DL-allantoin in combination with halite. While allantoin in a soluble form is often found in insect tissues and excreta; being present as a crystal, especially in such a large form, is curious and raises some interesting questions. More research is warranted to further understand mechanisms associated with such crystal formation in *B. hilaris* and can lead to a better understanding of the excretory process in this species and the role allantoin plays in the elimination of excess nitrogen.

Key words: nitrogen excretion, uric acid, X-ray diffraction

Bagrada hilaris (Burmeister) (Heteroptera: Pentatomidae), commonly known in its native range of Africa, India, and Asia as bagrada bug or painted bug, is a polyphagous herbivore recently reported as an invasive pest in the United States. Since its first detection in 2008, its distribution has expanded rapidly and it is now found as far north as central California and southern Nevada, east to western and central Texas, and south to northern areas in Mexico (Reed et al. 2013, Sánchez-Peña 2014, Torres-Acosta and Sánchez-Peña 2016). *B. hilaris* exhibits similar traits to many other herbivorous stink bugs (e.g., *Nezara viridula* or *Chlorochroa uhleri*), having five nymphal stages and feeds using piercing/sucking mouthparts which result in circular chlorotic lesions on host plant tissues (Reed et al. 2013, Palumbo et al. 2016). However, this species differs physically and most noticeably by its smaller size (~6 × 3 mm—a third that of *N. viridula*) and aposematic coloration in the adult stage as well as the nymphs (Taylor et al. 2015). The barrel-shaped eggs of *B. hilaris* are whitish or cream-colored when first deposited and turn pink to orange-red as the embryo develops. Females tend to oviposit few eggs per cluster and deposit them often in the soil (Taylor et al. 2015). Single females may produce 100–200 eggs during an average adulthood of 3–4 weeks (Singh and Malik 1993). All stages may be observed in large aggregations of hundreds of individuals (Reed et al. 2013) on both preferred and nonpreferred host plants and populations can reach outbreak levels very rapidly under very warm, dry environmental conditions when food hosts are available. The entire life cycle from egg to adult can be completed within two weeks under very warm conditions. The life history, distribution, and agricultural importance of *B. hilaris* in the southwestern United States have recently been reviewed (Palumbo et al. 2016). While it prefers both wild and cultivated

mustards, it has been shown to feed and damage a variety of other plants including corn and sunflower (Reed et al. 2013).

Insects exhibit a variety of mechanisms to excrete excess nitrogenous compounds which are mainly derived from amino acid and purine metabolism. This includes ammonia, which is primarily confined to aquatic insects where the toxicity of ammonia requires the use of copious quantities of water for detoxification (Chapman 2013, Pant 1988). Other excreted nitrogenous compounds include uric acid, allantoin, allantoic acid, hypoxanthine, xanthine, and some amino acids (Bursell 1967, Pant 1988). Urea seems to occur as a major excretory compound in only a few insect species.

Most terrestrial insects eliminate excess nitrogen by production of uric acid via the Malpighian tubule-rectum complex (Pant 1988, Cochran 1975). Uric acid, which is relatively water insoluble, can be eliminated as a solid pellet; the need to add water is minimal. Several species of plant sucking Heteroptera excrete substantial amounts of urea, a highly soluble compound. Because these species ingest excess water due to their feeding mechanism, i.e., sucking fluids from internal plant tissues, these insects have no urgent requirement to conserve water (Powles et al. 1972). Uric acid can be oxidatively degraded to allantoin and has been identified in the excreta of several species (Pant 1988, Kwangsoo et al. 2007). The cotton stainer, *Dysdercus fasciatus* (Heteroptera: Pyrrhocoridae) primarily produces allantoin as a nitrogenous waste product (Berridge 1965). The southern green stink bug, *N. viridula* (Heteroptera: Pentatomidae), a polyphagous pest, also produces allantoin as a waste product (Powles et al. 1972). In addition, the monophagous shield bug, *Parastrachia japonensis* Scott (Hemiptera: Parastrachiidae) has been shown to utilize allantoin as an excretory product (Kashima et al. 2006).

During the course of dissecting *B. hilaris* for studies designed to relate ovarian morphology to egg production or to determine sperm health, unique crystals were observed in both the midgut and oviducts. The objective of this research was to identify these crystals.

Materials and Methods

Insect Culture

B. hilaris adults of various ages were hand collected and brought into the laboratory at the USDA-ARS Facility at Stoneville, MS to develop colonies for various experimental research endeavors. Sites where the various individuals were collected included near the University of California campus (Riverside, CA), Gustine, CA, or Dixon, CA. Individuals were collected from a variety of different plant species including London rocket (*Sisymbrium irio* L.), shortpod mustard (*Hirschfeldia incana* (L.) Lagr.-Foss.), wild radish (*Raphanus raphanistrum* L.), or sweet alyssum (*Lobularia maritima* L.). Insects were primarily raised in plastic or screen cages and fed varying combinations of organic broccoli florets (*Brassica oleracea* L. Italica group) or seedlings of other species in the Brassicaceae. Plants were replaced with fresh material on an as needed basis in most instances. Paper toweling or strips of cotton material was placed in the cages to act as an oviposition substrate. For those individuals used for the determination of ovarian morphology to number of eggs produced, diet and holding conditions were more defined. In this case, adults were placed in round plastic containers (15 cm diam., Tri-State Plastics, Inc., Covington, KY) with multiple openings covered with fine brass mesh to provide ventilation without escape. Each container was lined with white paper toweling to provide a substrate for oviposition. Organic broccoli florets, supplied by local supermarkets, were provided as food and dietary moisture and were replenished every 24 h. Eggs were collected daily and nymphs were separated from adults and allowed to develop in large plastic containers. Fifth instar nymphs were placed individually into small (5 cm diam.) paper-lined and ventilated Petri dishes for daily observation of adult eclosion. Upon eclosion, sex was determined and one female and two males of similar age were placed in small Petri dishes. Mating groups were fed broccoli florets daily and observed for egg deposition and adult mortality. Periodically, typically every other day, the number of eggs were counted for each pair. All insects were maintained in an environmentally-controlled incubator (Percival-Scientific, Perry, IA) set at 30 ± 1 °C, 25–35% RH, and a 14:10 (L:D) h photoperiod.

Insect Dissections

In most cases individuals were selected at random from any number of colonies. For those obtained from the oviposition study live adults of known age and reproductive maturity were sent to the USDA-ARS Stoneville, MS facility for dissection and observation. Females were selected randomly and pinned dorsal side up to bees wax coated SEM stubs. The wings and pronotum of each were removed, and remaining tissues were covered with phosphate buffered saline (P. No. P4417, Sigma–Aldrich, Saint Louis, MO). The dorsal abdominal cuticle was removed and the internal organs were examined under a stereo microscope (Model No. M165C, Leica Microsystems, Buffalo Grove, IL) at various magnifications. In total, 75 individuals (44 females, 25 males, and 7 nymphs), with internal organs containing unique crystalline structures, were examined and the organs were removed for further characterization.

Crystal Analysis

Crystals were removed from internal organs using fine-tipped forceps and placed into 0.5 ml glass vials and sent to the X-ray Diffraction Laboratory in the Department of Chemistry, Texas A&M University College Station, TX for identification. A suitable crystal was selected and analyzed on a Bruker Micro-Source CMOS diffractometer (VENTURE by Bruker AXS, Madison WI). The crystal was kept at 102.38 K during data collection. Using Olex2 software (Dolomanov et al. 2009), the structure was solved with the ShelXT (Sheldrick 2015a) structure solution program using Direct Methods and refined with the ShelXL (Sheldrick 2015b) refinement package using Least Squares minimization. Unknown crystal structure was compared with known crystal data and the structure of $C_4H_6N_4O_3$ DL-allantoin (Mootz 1965). Needle-shaped crystals (powder) were identified by comparison to a calculated pattern of DL-allantoin.

Results and Discussion

Both acicular (i.e., needle-like, slender, and/or tapered) and cubic (i.e., cube shaped) crystals were observed in six of 75 individuals examined (8.0%). Crystals of either type were observed in higher numbers in females (6.7%), followed by males (1.3%) with no crystals observed in the minimal number of nymphs examined (0%) (Fig. 1). Crystals of both types were detected in the midgut and lateral oviducts of the females and midgut in males (Fig. 2a–d). The acicular crystals often appeared as distinct bundles (Fig. 2c). Although age of the males was unknown, females from the ovarian development study varied in age (from 20 days to 35 days postadult eclosion) and all were or had been reproductively active. The cubic crystals were identified as allantoin (Fig. 3) although the acicular crystals were most likely DL-allantoin in combination with halite (NaCl) (Fig. 4). Crystals varied in size with the acicular crystals ranging from 0.12 mm to

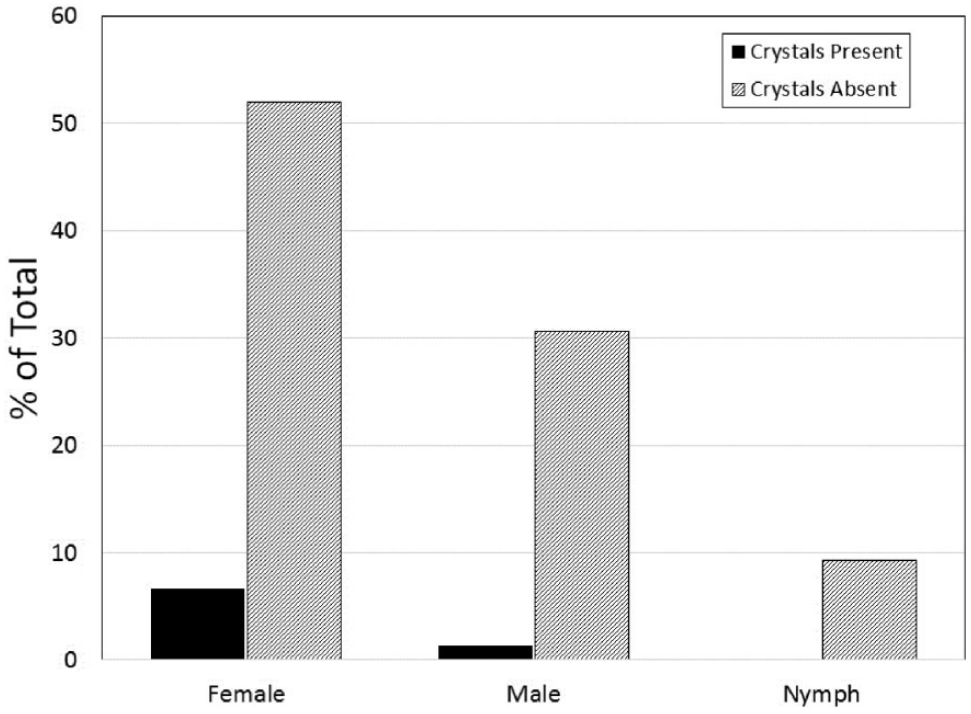

Fig. 1. Percentage of the total number of individuals examined having crystals of allantoin internally by sex and life stage.

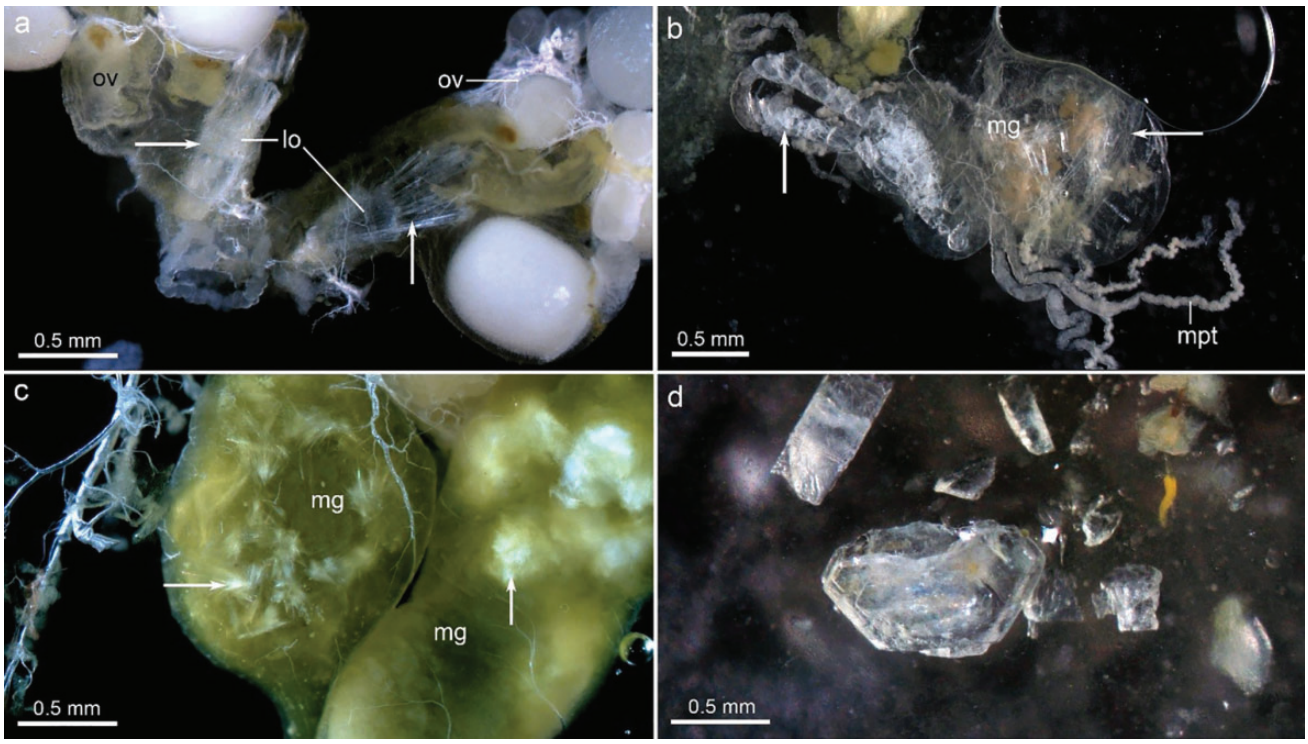

Fig. 2. Both acicular and cubic crystals identified in *B. hilaris* lateral oviducts (a) and mid gut (b, c). Crystals removed from midgut (d). ov – ovarioles, lo – lateral oviducts, mg – mid gut, mpt – Malpighian tubules.

0.5 mm in length although the cubic crystals ranged in length from 0.25 mm to over 1.0 mm with widths of ~0.25 mm.

The presence of such large and conspicuous crystals is apparently unique. Although crystals of uric acid have been observed in the Malpighian tubules of *Rhodnius prolixus* (Heteroptera: Reduviidae) (Wigglesworth 1931), we can find no mention in the literature of allantoin crystals being present internally in the Heteroptera or other insects. While allantoin is often found in insect tissues and excreta (Chapman 2013, Pant 1988) being present as a crystal, especially in such a large form, is curious and raises some interesting questions.

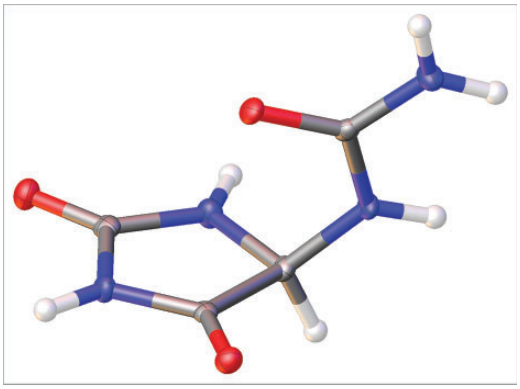

Fig. 3. Crystal structure of allantoin.

Mechanisms for the formation of such crystals in *B. hilaris* are unknown but the mechanics behind crystallization is fairly straightforward though a variety of factors can be influential (Mersmann 2001). For crystals to form, it is necessary for the soluble material (i.e., solute—in this case allantoin) to be present in a concentration high enough to increase the likelihood of allantoin molecules encountering one another allowing them to combine. When enough of these molecules combine they can form a nucleation site. Barring any outside forces, these precrystals fall out of solution, fostering the opportunities for increasing crystal size by additional accumulation. Excretory allantoin is normally highly soluble; significantly higher than uric acid. The solubility of allantoin (60 mg/100 ml) is approximately ten times that of uric acid (6 mg/100 ml) as reported by Bursell (1967) while Yalkowsky et al. (2016) indicated the allantoin is 87 times more soluble (522 mg/100 ml) as compared with

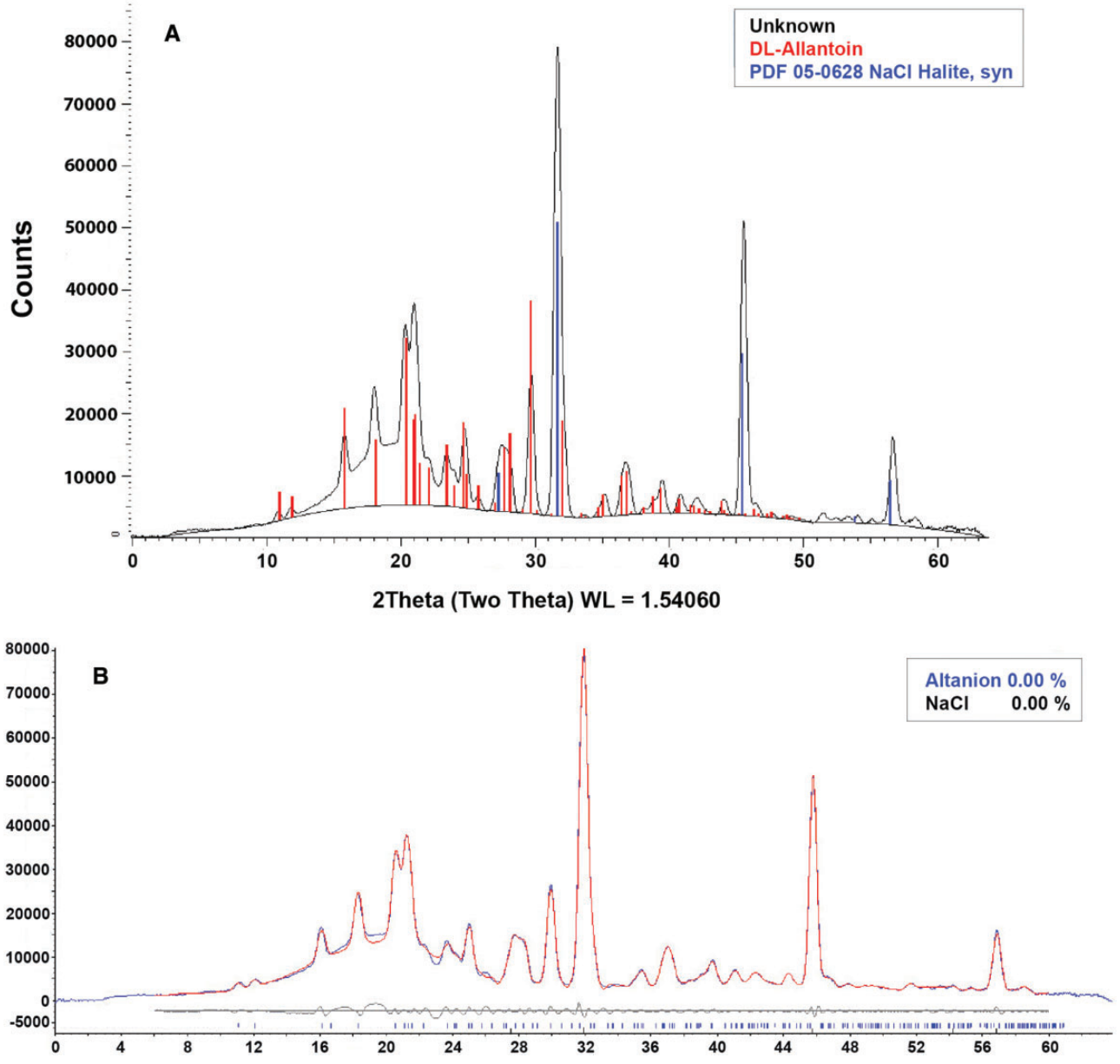

Fig. 4. Comparison of the unknown powder to the calculated pattern of DL-allantoin (a). Pawley refinement of the powder pattern with the unit cell of DL-allantoin and halite (NaCl; b).

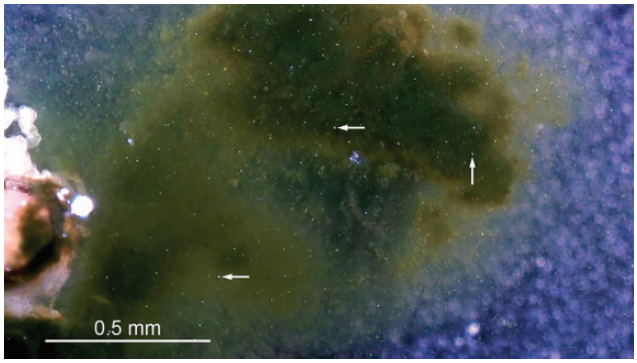

Fig. 5. Small reflective, crystalline-like particles in midgut (25×).

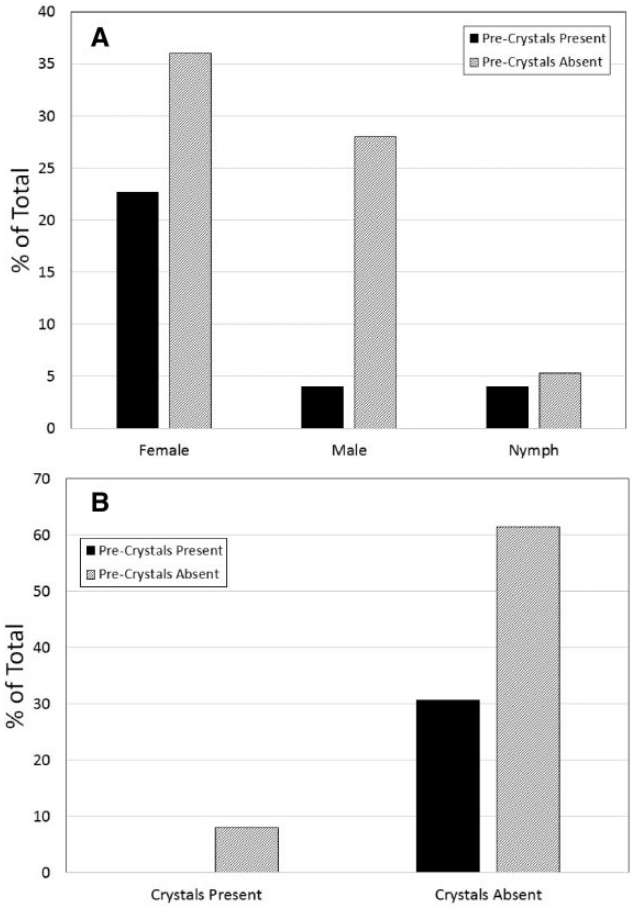

Fig. 6. Percentage of the total number of individuals examined with potential precrystals of allantoin in midgut by sex and life stage (a). Percentage of the total number of individuals examined with potential precrystals of allantoin in relation to the presence of actual allantoin crystals (b).

uric acid (6 mg/100 ml). Interestingly, we often observed small reflective particles floating in the mid-gut material that could possibly be precrystals of allantoin (Fig. 5). These putative precrystals were common and in the present study we observed 31.9% of the individuals having such reflective particles associated with the midgut material. Such precrystals were more commonly identified in females (22.7%) with approximately equal number of males and nymphs found to have the precrystals (4.0%; Fig. 6a). The putative precrystals were never identified in those individuals when allantoin crystals were present (Fig. 6b). The presence of the crystals (and potentially the reflective precrystals) suggests that allantoin may have been in very high concentrations in the midgut and oviducts.

Reasons for the possible increase in soluble allantoin in the midgut are unknown. Diet may have been a factor with increased nitrogen levels in the food stuff and/or decreases in water content, both of which may have contributed to increased allantoin levels. Allantoin is found in various plant species such as the Brassicaceae (Li 2008) evidently formed during periods of stress. One example is *Arabidopsis thaliana* (Arabidopsis), a member of the mustard family, where a variety of stressful conditions allowed an increase in allantoin levels (Takagi et al. 2016). These include drought, nutrient deficiencies, pathogen invasion, or increased saline conditions, among others. One reason may have been, in the case of the females that they were nearing the end of their reproductive life cycle and decreased egg formation may have increased nitrogenous waste levels through resorption of the immature follicles. Another possible factor could be the presence of gut symbionts that can process urea or uric acid to allantoin. Vogels and Van der Drift (1976) discussed the myriad ways bacteria and yeasts have been shown to contain the necessary enzyme systems for such oxidative degradation to allantoin. Donnellan and Kilby (1967) identified specialized cells in the fat body of *Periplaneta americana* (American cockroach) containing symbiotic bacteria that converted urates to several degradative products including allantoin. However, more recently, Sabree et al. (2009) showed that *Blattabacterium* sp. a gut symbiont in *P. americana* lack uricolytic capabilities. *Candidatus Walczuchella monophlebidarum*, the flavobacterial endosymbiont of *Llaveia axin axin* Llave (giant scale insect) was shown to aid in the formation of allantoin (Rosas-Pérez et al. 2014). Metagenomic analysis indicated that in *Dactylopius coccus* Costa (carmine cochineal scale insect) fungal symbionts play a key role in urate metabolism (Vera-Ponce de León et al. 2016).

Allantoin crystals have only been identified in only small proportion of the Bagrada bugs examined in this study. Apparently, it is not a common occurrence. In addition, the crystals have only been identified in laboratory reared individuals though the number of field collected individuals which were dissected immediately or in only a few days after capture is low. It is unknown if crystal formation occurs in field individuals; but we believe that the presence of crystals may be detrimental since they were found in the oviducts accumulating to the point of blockage. This is also based on the observation that the colony did not appear to be at its best during the time of this experiment. It is not clear if this was due to rearing conditions (use of broccoli florets only), a function of possible inbreeding depression or if these insects perform much better when they are reared on whole plants and when given a variety of both leafy and head-forming brassicas in large insect dorms. More research is warranted to understand those factors that favor crystal formation. Such information may allow for a greater understanding of the excretory process in this species and the role allantoin has in the metabolism and excretion of nitrogenous wastes.

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