Bursicon Expression May Reveal a Division Between Hemimetabolous and Holometabolous Insects

Logan D. Shirley and Hans-Willi Honegger

KEYWORDS. Bursicon, hemimetabolous, holometabolous

BRIEF. Fourteen species of insects were dissected to determine if the expression of the neurohormone bursicon in their adult stages correlated with their mode of metamorphosis.

ABSTRACT. All insects need to regularly shed their old shell to properly grow. The hormone bursicon hardens and darkens the new, soft protective exoskeleton. Once an insect becomes an adult, they no longer grow and do not shed their shell; therefore, they should not need bursicon. The research examined whether other insects support the hypothesis that hemimetabolous insects that change little while developing, like cockroaches, retain bursicon into adult stages, while holometabolous insects that change much, like butterflies, lose bursicon. Bursicon has been found in adult hemimetabolous insects, but not in adult holometabolous insects. Further research into bursicon expression could assist in pesticide development, since any chemical that disrupts bursicon's ability to harden and darken an insect's shell would leave it vulnerable. Fourteen species of insects were tested for bursicon. First, the insects' nervous systems were removed. They were incubated in antibodies against bursicon that were coupled to a fluorescent dye that allowed bursicon to fluoresce green. Therefore, through confocal microscopy, the cells that expressed bursicon fluoresced green. All hemimetabolous insects tested retain bursicon as adults. However, some holometabolous insects expressed bursicon, while others did not. More insects should be tested for bursicon to determine another reason for bursicon expression dichotomy.

INTRODUCTION.

Insects must occasionally discard their old shell because they are growing too large for it. The old shell must be shed and the newly secreted shell must harden. Bursicon, an insect hormone, hardens and darkens the soft new shell [1]. It also regulates inflation of the wings [2]. Bursicon is found in all insects and other arthropods [3]. Adult insects do not grow or shed their old shell anymore, so bursicon should not be needed in adult insects, as it would be detrimental.

As a hormone, bursicon is expressed in the insect nervous system, which consists of a nerve cord running through the middle of the insect body, connecting groups of nerve cells called ganglion [4]. Nerves extend from the ganglia to release hormones throughout the body. Bursicon is expressed in two large nerve cells in each ganglion in the abdomen and thorax, one on the right side and one on the left side, as shown in Figure 1 [5].

Crustacean cardioactive peptide, or CCAP, is another hormone present in insects [6]. It is present in all cells that produce bursicon, as well as in a second cell adjacent to each of these cells, so it is often used as a control in bursicon experimentation [2].

For holometabolous insects like the tobacco night hawk moth and the fruit fly that change much while developing, bursicon levels increase until the adult stage, then decrease significantly, as expected since bursicon is not needed in adult insects [7]. However, for hemimetabolous insects like the cockroach and the Australian cricket that change little while developing, bursicon levels are constant through adult stages [7].

The hypothesis was that holometabolous insects would not express bursicon in their adult stages, while hemimetabolous insects would express bursicon their entire lives. Further research into bursicon expression could assist in pesticide development; any chemical that could disrupt bursicon's ability to harden and darken a young insect's cuticle would leave the insect lacking camouflage and vulnerable to desiccation and predators before the insect can reproduce.

A side project in the lab explores the possibility that bursicon is involved in insect longevity. Hemimetabolous insects, with bursicon present throughout their entire lives, live much longer than holometabolous insects. To investigate whether bursicon contributes to that difference, hemimetabolous and holometabolous insects' bursicon levels will be compared. A second way to examine this possibility is to compare queen and worker bees' bursicon, since queen bees live years longer than worker bees.

MATERIALS AND METHODS.

Dissection.

In order to determine the presence of bursicon in an insect, the insects were first dissected, and the ventral nervous system was removed. The basic steps are similar in all insect dissections. First, the insect was placed in a cold room for thirty minutes or until it was slow enough to catch. After anesthetizing the insect by placing it in a -20°C freezer, it was killed via a pushpin through the head and cuts through the ventral cuticle to the left and right of the nervous system. Next, the cuticle was lifted and scraped the muscles, nervous system, and other organs from the underside of the cuticle. Finally, the cuticle was cut off. Now, the nervous system could be accessed and cut out. Removal of accessory organs like muscles and tracheal tubes from the nervous system was often necessary.

Tissue Fixation.

It was then necessary to fix the tissue in order to prevent the hormones from degrading. For this process, the nervous system was incubated in a special fixative called Bouin's fixative for a number of hours, depending on the size of the nervous system. The nervous system was then moved to 70% isopropanol, where it stayed for at least two hours but could stay for multiple days at a temperature of 4° C. The nervous system was then incubated in collagenase, which creates holes in the tissue sheath of the ganglia. This effectively breaks down the barrier surrounding the ganglia and allows the antibodies to reach the nerve cells more easily. After, the nervous system was pre-incubated in a normal goat serum (NGS) solution. NGS ensures that the antibodies bind only to the target regions, bursicon and CCAP.

Immunocytochemistry.

Conventional immunocytochemistry was then performed by incubating the nervous system in two different primary and secondary antibodies. The nervous systems were incubated in primary antibodies against pburs, one of the two monomers of bursicon, and against CCAP. CCAP was used as a control, since it is expressed in some of the same neurons as bursicon. The secondary antibodies were coupled to the fluorescent dyes, or fluorophores, that allowed pburs to fluoresce green and CCAP to fluoresce red. Both antibodies were needed because the fluorophores are coupled only to the secondary antibodies.

The nervous system was incubated in the primary antibody over two days, then incubated in the secondary antibodies overnight. All antibody incubation was performed at 4° C. Finally, the nervous system was embedded on a slide in a water-soluble mounting medium, where it can stay without significant degradation for months.

Visualization.

Finally, a ZEISS 510 laser scanning confocal microscope was used to analyze the fluorescence in the nervous system. Pupae, larvae, and adult or nymph and adult for holometabolous and hemimetabolous insects, respectively, were analyzed for as many species as could be acquired. At least one individual of each of fourteen species, including the cockroach, 13-year cicada, dragonfly, locust, lubber grasshopper, seedcorn beetle, murky ground beetle, Japanese beetle, caterpillar hunter, eastern firefly, soldier beetle, stag beetle, carpenter bee, and honeybee were obtained.

RESULTS.

American Cockroach.

The scanning confocal microscope pictures verify that the hemimetabolous cockroach, *Periplaneta americana*, expresses both bursicon and CCAP (Fig. 1). CCAP is expressed in a pair of cells on both sides of each thoracic ganglion and the first three abdominal ganglia; it is not expressed in the terminal ganglion. Bursicon is expressed in only one of each of these pairs of cells, but it is expressed in all of the same ganglia as CCAP. CCAP and bursicon projections are also visible throughout each of the ganglia.

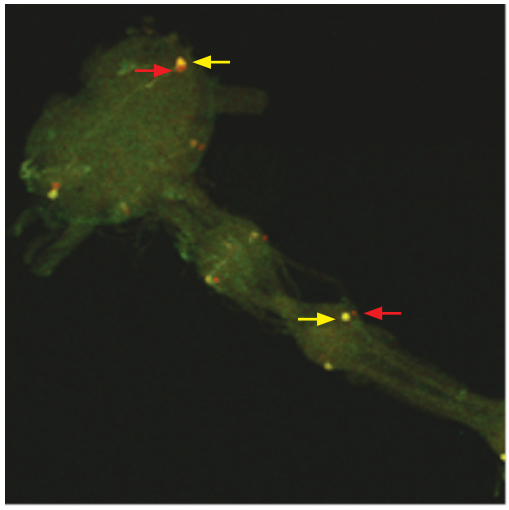

Figure 1. The last thoracic and first two abdominal ganglia of an adult hemimetabolous cockroach. Cells expressing CCAP are visible in red, cells expressing bursicon are visible in green, and cells that express both are visible in yellow. Cockroaches express both CCAP and bursicon.

The expression patterns for nymphal and adult cockroaches are the same; therefore, cockroaches express bursicon in all developmental stages. Since reliable pburs and CCAP labeling was obtained in *P. americana,* its nervous sytem was used as positive control in all other insect dissections.

Eastern Firefly.

The adult holometbolous eastern firefly, of the genus Photinus, shows expression of both CCAP and bursicon in scanning confocal microscope pictures (Fig. 2). The expression pattern appears to be similar to the dragonfly and cockroach expression patterns, with both sides of the ganglion containing one solely CCAP-expressing cell and one CCAP- and bursicon-expressing cell, yielding a pair on each side of the ganglion. In at least one abdominal ganglia, there were also two pairs of only CCAP-expressing cells in the center of the ganglion. Projections of both hormones are common, but appear to be mostly constrained to the sides of the ganglion, where the cells are present.

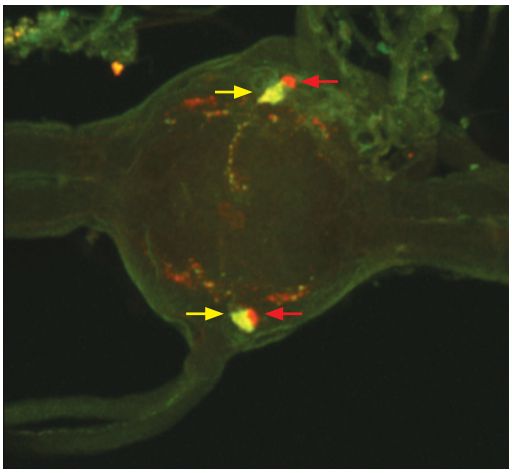

Figure 2. The abdominal ganglion of an adult holometabolous eastern firefly, expressing both CCAP and bursicon in two cells (yellow arrows) and only CCAP in two other cells (red arrows).

Honeybee.

The scanning confocal microscope pictures of the adult holometabolous worker bee, *Apis mellifera,* confirm that bursicon is not present (Fig. 3). CCAP, however, is expressed in at least four cells in each thoracic and abdominal ganglion.

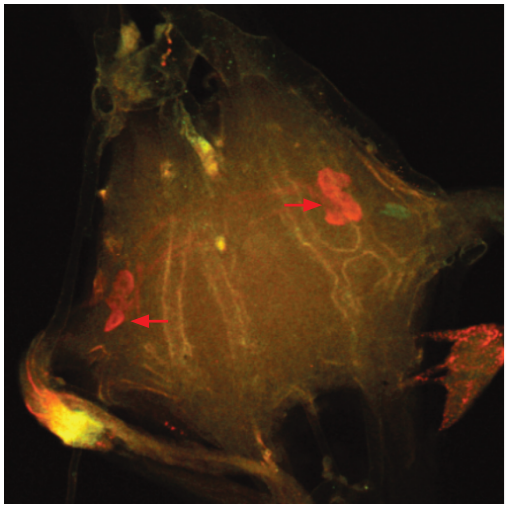

Figure 3. The third abdominal ganglion of an adult holometabolous worker bee. It expresses only CCAP, indicated by the red arrows.

The adult holometabolous queen bee expresses only CCAP, but it is expressed in at least six cells in each thoracic and abdominal ganglion. Larval bees express only CCAP also, and they express it in one cell on both sides of each thoracic and abdominal ganglion. The bee pupa expressed CCAP in at least five cells in every thoracic and abdominal ganglion, but again, did not express bursicon. Very few projections are visible in any stage.

DISCUSSION.

Multiple insects of both major distinctions were tested: holometabolous insects that use complete metamorphosis and hemimetabolous insects that use incomplete metamorphosis. The hypothesis was that holometabolous insects would lose bursicon in the adult stages, while hemimetabolous insects would retain bursicon throughout their lives, based on previous research and the fact that insects should no longer need bursicon after their last ecdysis.

The holometabolous insects tested included the honeybee, carpenter bee, Japanese beetle, murky ground beetle, caterpillar hunter, eastern firefly, soldier beetle, stag beetle, and seedcorn beetle (Supplementary Figs. S1-S11). None of these insects were expected to express bursicon, in concordance with previous research, but the majority of the insects did express bursicon in their adult stages. All of the hemimetabolous insects tested expressed both CCAP and bursicon, as expected. Therefore, the original hypothesis was proven incorrect. There is also little evidence to suggest a secondary cause of bursicon expression dichotomy.

The particular instance of the Japanese beetle is interesting to examine (Supplementary Fig. S7). The three individual beetles tested exhibited bursicon in varying numbers of cells. The hypothesis was that the Japanese beetle adult loses bursicon expression as it ages and that beetles of three different ages were caught. Since only one of any of the other beetle species was caught, it is possible that all beetles lose their bursicon expression as they age.

Another interesting result was the lack of bursicon in any stage of the honeybee, *Apis mellifera* (Fig. 3). Some bursicon in larval and/or pupal stages was expected, when the insect would still need bursicon to harden and darken its cuticle after ecdysis. However, no bursicon was found in any stage. There are a few possible reasons for this unexpected result; the insect may express bursicon in neurons other than those which were tested, such as the brain or subesophageal ganglion. They may also express it in such a small time window that none of the insects tested were in that life stage. Most heretically, honeybees may not use bursicon at all to harden and darken their new, soft cuticle after ecdysis.

A secondary project within the broader research was the potential link between bursicon expression and insect longevity. For this research, bursicon expression between queen and worker bees was compared, since, as in most social insects, queens live years longer than workers. However, no bursicon expression was found in bees, which indicates that bursicon might not support longevity.

The new data concerning holometabolous insects that express bursicon in their adult stages is novel and could change the field. Few papers consider a secondary reason for bursicon expression dichotomy, the first explanation being the original hypothesis. The lack of a secondary explanation could be mainly due to lack of knowledge of bursicon activities. Bursicon's currently known functions do not account for its presence in insects' adult stages. Learning bursicon's secondary function in adult insects could help us discover why only some holometabolous insects express it.

Determining which insects express bursicon could potentially be useful in pesticide development. Learning which insects still use this particular hormone during their adult lives could help us develop a pesticide to target specific species of insects, without environmental harm. If the insect still expresses bursicon in their adult stages, a bursicon pesticide could affect these insects at all stages of their life, effectively killing off not only young insects that are still ecdysing, but also potentially reproductive adults. However, the pesticide would not affect the adults of those species that do not express bursicon as adults. This could be useful if certain species are beneficial to one's needs, while others are not.

For example, a bursicon pesticide could be used in the recent and harmful reappearance of the bedbug. Since bedbugs are hemimetabolous, the hypothesis is that they express bursicon as adults, and a bursicon-targeting pesticide could kill the insects through chemical means, without any environmental effect. It would stop the insects' ability to harden and darken their cuticle after ecdysis, effectively killing them. It would affect only insects that express bursicon, like cockroaches, which are also unwanted.

It is important to note that fewer hemimetabolous insects, five, were tested than holometabolous insects, nine. Given more time, further testing would have been performed with a wider variety of insects, not only to determine whether any hemimetabolous insects exist that do not express bursicon, but also to determine if the beetles are the only order of holometabolous insects that express bursicon, as is supported so far. Also, the evolutionary history of all of the holometabolous insects tested so far should be analyzed, in order to determine if the dichotomy of bursicon expression is of an evolutionary origin. Alternative reasons for bursicon expression should also be more extensively researched.

Further bursicon research could not only discover a reason for the holometabolous dichotomy of bursicon expression, but also a way to predict which insects express bursicon. While the current bursicon findings have already changed our present thoughts on this intriguing neurohormone, there is still much more to be learned. Additional studies could unveil more of bursicon's secrets and its applications for improving life.

ACKNOWLEDGMENTS. I would like to thank my PI, Dr. Honegger, for his constant guidance, support, and assistance. I would like to thank my School for Science and Math instructors, Dr. Creamer, Dr. Vanags, and Dr. Eeds, for all their support and guidance. I would like to also thank those who brought in samples for our experiments, including the School for Science and Math Class of 2013.

SUPPORTING INFORMATION.

Figure S1. *Magicicada tredecim* nervous system and results.
Figure S2. *Anax junius* nervous system and results.
Figure S3. *Locusta migratoria* nervous system and results.
Figure S4. *Romalea guttata* nervous system and results.
Figure S5. Seedcorn beetle nervous system and results.
Figure S6. *Harpalus caliginosus* nervous system and results.
Figure S7. *Popillia janpoica* nervous system and results.
Figure S8. *Calosoma scrutator* nervous system and results.
Figure S9. Soldier beetle nervous system and results.
Figure S10. *Lucanus elaphus* nervous system and results.
Figure S11. *Xylocopa virginica* nervous system and results.

REFERENCES.

1. G. Fraenkel, C. *Hsiao, J. Insect Physiol.* **11**, 513 (1965).
2. E. M. Dewey, *et al., Curr. Biol.* **14**, 1208 (2004).
3. T. Van Loy, *et al., Gen. Comp. Endocr.* **153**, 59 (2007).
4. C. W. Luo, *et al., PNAS.* **102**, 2820 (2005).
5. L. Dai, *et al., J. Comp. Neurol.* **506**, 759 (2008).
6. J. Ewer, S. E. Reynolds, *Hormones, Brain, and Behavior.* (Academic Press, New York, 2002).
7. H. W. Honegger, E. M. Dewey, J. Ewer, *J. Comp. Physiol. A.* **194,** 989 (2008).

Logan Shirley is a student at McGavock High School and enrolled in the School for Science and Math at Vanderbilt.