6-2020

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The Canadian Entomologist

Dissection of *Anoplophora glabripennis* (Coleoptera: Cerambycidae) larval tissues for physiological and molecular studies

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Abstract

Many biological processes are partitioned among organs and tissues, necessitating tissue-specific or organ-specific analysis (particularly for comparative -omics studies). Standardised techniques for tissue identification and dissection are therefore imperative for comparing among studies. Here we describe dissection protocols for isolating six key tissues/organs from larvae of the Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky) (Coleoptera: Cerambycidae): the supraoesophageal ganglion, posterior midgut, hindgut, Malpighian tubules, fat body, and thoracic muscle. We also describe how to extract haemolymph and preserve whole larvae for measurements such as protein, lipid, and carbohydrate content. We include dissection protocols for both fresh-killed and previously frozen specimens. Although this protocol is developed for *A. glabripennis*, it should allow standardised tissue collection from larvae of other cerambycids and be readily transferrable to other beetle taxa with similar larval morphology.

Résumé

Les processus physiologiques et moléculaires ont cours au sein de différents tissus, ce qui nécessite pour chacun des analyses distinctes, en particulier dans le cadre d'études «omiques» comparées. Il est donc impératif de standardiser les techniques de dissection et les protocoles d'identification des tissus pour comparer des études. Nous décrivons ici les protocoles de dissection pour isoler six tissus ou organes clés de larves du longicorne asiatique *Anoplophora glabripennis* (Motschulsky) (Coleoptera: Cerambycidae), soient le ganglion supraoesophagien, l'intestin moyen, le tube digestif postérieur, les tubules de Malpighi, le corps gras et les muscles thoraciques. Nous décrivons également comment extraire l'hémolymphe et conserver les larves entières pour effectuer des mesures de teneur en protéines, en lipides et en glucides. Nous incluons des

protocoles de dissection non seulement pour les échantillons frais, mais aussi pour les échantillons congelés, afin d'aider les chercheurs qui n'ont pas accès à des spécimens vivants. Bien que ces protocoles aient été élaborés pour *A. glabripennis*, ils devraient permettre de standardiser la collecte de tissus chez les larves d'autres cérambycidés, et être aisément transférables à d'autres taxons de coléoptères avec une morphologie larvaire similaire

Introduction

Insects maintain homeostasis via integrated physiological systems with a division of labour among tissues (Klowden 2013). Thus, most physiological processes should be explored at the tissue level; for example, the midgut is usually the focus of digestion and absorption studies (Caroci and Noriega 2003; Jagadeshwaran *et al*. 2010), while the Malpighian tubules are normally responsible for excretion (Dow *et al*. 1998). However, many transcriptomic studies analyse homogenised whole insects (*e.g.*, Teets *et al*. 2012; Poupardin *et al*. 2015; MacMillan *et al*. 2016; Torson *et al*. 2017; Koniger and Grath 2018), and thus do not capture tissue-specific processes, which may be important. For example, upregulated sodium pump expression in the Malpighian tubules would likely reduce primary urine production, while a similar upregulation in the hindgut would likely enhance water reabsorption to the haemolymph. The physiological implications of these two scenarios would not be predicted from a whole-animal homogenate (Des Marteaux *et al*. 2017, 2018).

Shifting from whole animal to tissue-specific analyses must be done carefully, as inconsistencies in dissection technique or inadvertent inclusion of non-target tissues (*e.g.*, inclusion of fat traces with gut samples) can lead to misleading results. Furthermore, tissues, organs, and organ systems may be improperly homologised. For example, the large, anterior portion of the midgut in cerambycid larvae has been misinterpreted as belonging to the foregut (Wei *et al*. 2006; Choo *et al*. 2007), likely leading to spurious reports of chitinase and cellulase enzymes occurring in the foregut. Thus, it is important to standardise dissection and sample preparation protocols to ensure comparable results across samples within a study as well as among studies on the same or related species. Such standardised tissue classification (and developmental

staging) protocols are *de rigeur* for model organisms (*e.g.*, Sinha 1958; Eaton 1974; Bainbridge and Bownes 1981; Goodman *et al*. 1985; Curtis *et al*. 1999).

Some cerambycid (longhorned) beetles are important forest pests (Eyre and Haack 2017). Asian longhorned beetle (*Anoplophora glabripennis* Motschulsky; Coleoptera: Cerambycidae: Lamiinae) larvae feed on healthy tree tissues across a broad host range (Faccoli *et al*. 2016) and this species has been inadvertently introduced in North America and Europe (Nehme *et al*. 2010; Dodds and Orwig 2011; Hull-Sanders *et al*. 2017). As with many economically important invasive species, the physiology and molecular biology of Asian longhorned beetle is the focus of current studies in multiple laboratories and countries. Thus, standardised dissection methods (currently unavailable for any cerambycid) are required to ensure appropriate tissue/organ identification and consistent sampling and comparisons.

Here we present a protocol for collecting haemolymph and dissecting six tissues/organs (the supraoesophageal ganglion, posterior midgut, hindgut, Malpighian tubules, fat body, and thoracic muscle) from Asian longhorned beetle larvae, distinguishing the appearance of the tissues in fresh and previously frozen specimens. This protocol should facilitate consistency among studies of Asian longhorned beetle and other cerambycid larvae and be useful for other beetles with similar larval morphology.

Protocol

Asian longhorned beetle larvae used in the present study originated from a laboratory colony derived from the Chicago (Illinois, United States of America) infestation, and were reared under quarantine (Canadian Food Inspection Agency authorisation WA-2013-017) at the Insect Production and Quarantine Laboratory at the Great Lakes Forestry Centre (stock number:

Glfc:IPQL:AglaUIC01; Great Lakes Forestry Centre, Sault Ste. Marie, Ontario, Canada) (Roe *et al*. 2018). We reared larvae at 25 ºC under constant darkness for 10 weeks and then exposed them to 7 ºC to induce a developmental arrest (Keena and Moore 2010). We sampled all larvae during this developmental arrest. We dissected live larvae at the Great Lakes Forestry Centre and freeze-killed larvae at the University of Western Ontario in London, Ontario. Freeze-killed larvae had been flash-frozen in liquid nitrogen at the Great Lakes Forestry Centreand thereafter stored at -80 °C until shipping to the University of Western Ontario on dry ice.

External larval anatomy

Asian longhorned beetle larvae are large, reaching up to 50 mm in length and weighing more than 1 g (Keena and Moore 2010). The external anatomy of the cylindrical, cream-coloured larvae (Fig. 1) has been described in detail elsewhere (Cavey *et al*. 1998) and is similar to that of other cerambycid larvae (Svacha and Lawrence 2014). The head is heavily sclerotised, prognathous, and partially retracted. The prothorax (Fig. 1: t1) is expanded relative to the mesothoracic and metathoracic segments (Fig. 1: t2 and t3) and has a partially-sclerotised pronotum. The mesothoracic spiracle is well developed and there are functional spiracles on the first eight abdominal segments (Fig. 1: a1–a8). The larvae lack both thoracic legs and abdominal prolegs: the virtual absence of thoracic legs distinguishes Lamiinae from other cerambycid subfamilies which (except for some Cerambycinae) have segmented legs.

Haemolymph extraction

Prior to dissection, we punctured the anterior edge of the prothoracic shield cuticle of live larvae with a 22-gauge 1-inch needle (Thermo Fisher Scientific; Sunnyvale, California, United

States of America) to create a bleeding wound (Fig. 2). We held the larva over a 1.5-mL tube to collect the haemolymph. The total volume of extracted haemolymph varied from 90–300 μL among individuals due to variation in larval size. We centrifuged the collected haemolymph for 3 seconds at 2000 × *g* to separate the haemolymph (infranatant) from lipids (supernatant) and other debris (high-density fraction). We used this low spin force to avoid separating haemocytes from the plasma of the haemolymph. We aliquoted known quantities of haemolymph for biochemical analyses into 1.5-mL microcentrifuge tubes and flash-froze them in liquid nitrogen. Evaporation should be minimised for some applications, such as measuring haemolymph osmolality. In these cases, we covered the haemolymph with a layer of mineral oil (M5904, Sigma-Aldrich,Oakville, Ontario, Canada) before freezing to reduce evaporation. Haemolymph will begin to melanise after 15 minutes (Fig. 2B), so it must be processed quickly. Because freezing damages tissue and perturbs homeostasis, hemolymph from previously-frozen larvae is probably not physiologically-relevant, so while extracted hemolymph can be frozen, we do not recommend extracting hemolymph from previously-frozen larvae.

Internal anatomy and dissection

Larval dissection. We dissected larvae in a dark-bottomed Sylgard-lined dish (Living Systems Instrumentation; Saint Albans City, Vermont, United States of America) that was deep enough (approximately 3 cm) to submerge the entire body in dissection media of choice (examples below). We pinned the larvae through the sides of the prothorax (Fig. 1: t1) and the last abdominal segment (Fig. 1: a10) to hold it ventral side-down with the body straight and the cuticle taut. We then cut through the cuticle along the dorsal midline anteriorly using microscissors held with the blades angled dorsally to avoid puncturing the gut. We spread the

120 body walls laterally with 8–10 additional pins to open the body cavity and expose the internal
121 organs (Fig. 3). To relax the tissues for easier dissection, frozen larvae were thawed and
122 submerged in an aqueous solution chosen based on specific downstream needs (*e.g.*, water,
123 Ringer's, or insect saline). Submerging fresh (unfrozen) specimens entirely in solution caused
124 the fat body to dislodge and obscure other tissues, therefore we pipetted 1–2 mL of an aqueous
125 solution (as above) into the opened body cavity to wash away the fat body and relax the tissues
126 of fresh specimens. We placed sampled tissues in a drop of fresh saline on a sterile Petri dish and
127 cleared the samples of fat body and large (readily visible) tracheae and nervous tissue prior to
128 flash-freezing in microcentrifuge tubes.
129
130 **Fat body, Malpighian tubules, and gut**. The fat body occupies much of the larval internal
131 cavity and obscures many internal organs, although the gut (orange or green in colour) is
132 typically at least partly visible. We collected fat body samples from three areas: the lateral
133 regions of the prothorax (Fig. 1: t3-a1); posterior midgut (Fig. 1: a4-a5); and hindgut (Fig. 1: a7-
134 a9). We removed a total of approximately 300–500 mg for metabolite analysis, then removed
135 and discarded all remaining fat body (an additional 300 mg could easily be collected from a
136 medium-sized larva). Removing the remaining fat improves visibility of the gut and other organs
137 (Fig. 4).
138 We collected the Malpighian tubules prior to removing the gut. The Malpighian tubules (which
139 may be entangled in tracheae) attach at the bulbous midgut-hindgut intersection (Fig. 5). Because
140 cerambycid larvae have cryptonephridial Malpighian tubules, we collected the free regions of the
141 tubules prior to their entrance into the rectal wall. At this stage of the dissection there are critical
142 differences between fresh and freeze-killed specimens. For freeze-killed larvae, the gut may be

pinned to one side away from the body to expose and isolate the Malpighian tubules. Multiple tracheae must be severed to liberate the gut, and this should be done carefully to avoid damaging the entwined Malpighian tubules (Fig. 5). Note that frozen larvae have white, rather than yellow, Malpighian tubules. Although the Malpighian tubules and tracheae both appear white in frozen larvae, the Malpighian tubules can be distinguished by their opaque, slightly flattened and repeatedly curved (bumpy) appearance, while the tracheae, being internally supported by the cuticular taenidia (visible with specific lighting and/or higher magnification), appear cylindrical, hollow, shiny, and without repeated curves (Fig. 5). Although we could not pin the extremely fragile midguts of fresh larvae to the side, the Malpighian tubules of fresh larvae were bright yellow and therefore easily distinguishable from the tracheae. We used fine forceps to sever the Malpighian tubules from the base at the hindgut-midgut intersection.

Morphological differences between the midgut and hindgut are clear in both fresh and frozen specimens; the junction between the gut regions is identifiable by a decrease in diameter, attachment of Malpighian tubules (Fig. 5) and (potentially) a change in colour (Fig. 4). To collect the hindgut, we severed the midgut-hindgut junction with microscissors. We sampled the posterior midgut by collecting a 2.0–2.5 cm section of gut anterior to the midgut-hindgut junction. After dissecting each gut section, we cut open the section, removed the solid contents (food bolus) with forceps, and then rinsed the tissue with fresh insect saline (note that a portion of the gut luminal microbiota will likely remain in the gut folds). Inclusion of the gut contents for downstream analyses (*e.g.*, microbiome; Scully *et al*. 2014) can be achieved by clamping the gut closed at each end prior to severing the tissue (see MacMillan and Sinclair 2011).

Muscle. Large muscle bundles are found in the prothorax and attached to the body wall of each thoracic and abdominal segment and are among the easiest tissue to sample. We sampled muscle bundles within the prothorax (*i.e.*, the main dorsal head retractor and pharyngeal muscles) (Fig. 6). These firm, striated muscles are easily distinguishable from other musculature in both fresh and frozen individuals. We used forceps or microscissors to remove prothoracic muscle (making cuts away from the midline), being careful to avoid damaging the nervous tissue such as the supraesophageal ganglia or nerve cord along the midline (see below).

Supraoesophageal ganglia. The brain (supraoesophageal ganglia) is comprised of two lobes placed above the oesophagus within the sclerotized cranium, which is retracted beneath the pronotum (Fig. 7). We used the oesophagus as a guide to locate the brain. First, we carefully exposed the oesophagus by pushing the posterior cranial margin up and forward. This helps to expose the brain, allowing access to it through the occipital foramen. To extract the brain, we gently severed the circumoesophageal nerve ring with microscissors or forceps and removed the brain with forceps.

Discussion

There are more than 36 000 described Cerambycidae species (Monne *et al*. 2017). Consistent dissection approaches, such as those outlined here, should facilitate tissue-specific physiological and molecular research for Asian longhorned beetle and related cerambycids even for non-morphologists. The large size of Asian longhorned beetle larvae allows tissue harvesting for multiple purposes (*e.g.*, samples destined for both physiological assays and genotyping), providing

an opportunity to associate physiology with population genetic information; an approach that can inform mechanistic species distribution models for invasive species (Buckley *et al*. 2010).

Haemolymph collection is perhaps the most time-dependent component of the protocol we describe. Melanisation in Asian longhorned beetle is fairly fast, so haemolymph should be sampled, processed, and stored within 15 minutes. Haemolymph samples destined for assays such as haemocyte counts or metabolite concentrations can be flash frozen in liquid nitrogen. For other downstream measurements (*e.g.*, haemolymph osmolality), it is necessary to store the samples under mineral/immersion oil or in a capillary tube to prevent evaporation. Note that freezing disrupts cellular integrity, thus haemolymph collected from previously frozen specimens may contain products from ruptured cells of various tissues. One potential solution to this problem would be to centrifuge at a higher speed to remove cells and large cellular debris.

We expect this protocol to be generally applicable throughout Cerambycidae, although species-specific differences in tissue size and appearance are likely. For example, because cerambycid larvae range from approximately 5–200 mm in length (Lawrence 1991) we expect that tissues of smaller individuals may be difficult to dissect and differentiate, and may not yield sufficient material for some downstream applications (*e.g.*, RNA-seq). In such cases, tissue samples from multiple individuals can be pooled. Larval morphology among beetle families can vary significantly, however the methods outlined here should be generalisable to families with similar morphology to Cerambycidae.

Acknowledgements

We would like to thank the technical staff in the Insect Production and Quarantine Laboratories at the Great Lakes Forestry Centre for their work in rearing specimens used for the development

of this protocol. We would also like to thank Petr Švácha for lending his expertise to improve earlier drafts of the manuscript. This work was supported by a Genome Canada, Genome British Columbia, and Genome Quebec [LSARP 10106; Genome Canada] grant for the Biosurveillance of Alien Forest Enemies (BioSAFE).

References

Bainbridge, S.P. and Bownes, M. 1981. Staging the metamorphosis of *Drosophila melanogaster*. Journal of Embryology and Experimental Morphology, **66**: 57–80.

Buckley, L.B., Urban, M.C., Angilletta, M.J., Crozier, L.G., Rissler, L.J., and Sears, M.W. 2010. Can mechanism inform species' distribution models? Ecology Letters, **13**: 1041–1054. https://doi.org/10.1111/j.1461-0248.2010.01479.x.

Caroci, A.S. and Noriega, F.G. 2003. Free amino acids are important for the retention of protein and non-protein meals by the midgut of *Aedes aegypti* females. Journal of Insect Physiology, **49**: 839–844. https://doi.org/10.1016/S0022-1910(03)00134-3.

Cavey, J.F., Hoebeke, E.R., Passoa, S., and Lingafelter, S.W. 1998. A new exotic threat to North American hardwood forests: an Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky) (Coleoptera : Cerambycidae). I. Larval description and diagnosis. Proceedings of the Entomological Society of Washington, **100**: 373–381.

Choo, Y.M., Lee, K.S., Kim, B.Y., Kim, D.H., Yoon, H.J., Sohn, H.D., and Jin, B.R. 2007. A gut-specific chitinase from the mulberry longicorn beetle, *Apriona germari* (Coleoptera: Cerambycidae): cDNA cloning, gene structure, expression and enzymatic activity. European Journal of Entomology, **104**: 173–180. https://doi.org/10.14411/eje.2007.026.

Curtis, N.J., Ringo, J.M., and Dowse, H.B. 1999. Morphology of the pupal heart, adult heart, and associated tissues in the fruit fly, *Drosophila melanogaster*. Journal of Morphology, **240**: 225–235. https://doi.org/10.3390/genes9120624.

Des Marteaux, L.E., Khazraeenia, S., Yerushalmi, G.Y., Donini, A., Li, N.G., and Sinclair, B.J. 2018. The effect of cold acclimation on active ion transport in cricket ionoregulatory tissues. Comparative Biochemistry and Physiology A, **216**: 28–33. https://doi.org/10.1016/j.cbpa.2017.11.005.

Des Marteaux, L.E., McKinnon, A.H., Udaka, H., Toxopeus, J., and Sinclair, B.J. 2017. Effects of cold-acclimation on gene expression in fall field cricket (*Gryllus pennsylvanicus*)

ionoregulatory tissues. BMC Genomics, **18**: article 357, 1–17. https://doi.org/10.1186/s12864-017-3711-9.

Dodds, K.J. and Orwig, D.A. 2011. An invasive urban forest pest invades natural environments - Asian longhorned beetle in northeastern US hardwood forests. Canadian Journal of Forest Research, **41**: 1729–1742. https://doi.org/10.1139/X11-097.

Dow, J.A.T., Davies, S.A., and Sozen, M.A. 1998. Fluid secretion by the *Drosophila* Malpighian tubule. American Zoologist, **38**: 450–460. https://doi.org/10.1093/icb/38.3.450.

Eaton, J.L. 1974. Nervous system of the head and thorax of the adult tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae). International Journal of Insect Morphology and Embryology, **3**: 47–66. https://doi.org/10.1016/S0020-7322(74)81006-8.

Eyre, D. and Haack, R.A. 2017. Invasive cerambycid pests and biosecurity measures. *In* Cerambycidae of the world: biology and pest management, *Edited by* Q. Wang. CRC Press, Boca Raton, Florida, United States of America. Pp. 563–607.

Faccoli, M., Favaro, R., Concheri, G., Squartini, A., and Battisti, A. 2016. Tree colonization by the Asian longhorn beetle, *Anoplophora glabripennis* (Coleoptera: Cerambycidae): effect of habitat and tree suitability. Insect Science, **23**: 288–296. https://doi.org/10.1111/1744-7917.12192.

Goodman, W.G., Carlson, R.O., and Nelson, K.L. 1985. Analysis of larval and pupal development in the tobacco hornworm (Lepidoptera, Sphingidae), *Manduca sexta*. Annals of the Entomological Society of America, **78**: 70–80. https://doi.org/10.1093/aesa/78.1.70.

Hull-Sanders, H., Pepper, E., Davis, K., and Trotter, R.T. 2017. Description of an establishment event by the invasive Asian longhorned beetle (*Anoplophora glabripennis*) in a suburban landscape in the northeastern United States. Public Library of Science One, **12**: e0181655. https://doi.org/10.1371/journal.pone.0181655.

Jagadeshwaran, U., Onken, H., Hardy, M., Moffett, S.B., and Moffett, D.F. 2010. Cellular mechanisms of acid secretion in the posterior midgut of the larval mosquito (*Aedes aegypti*). Journal of Experimental Biology, **213**: 295–300. https://doi.org/10.1242/jeb.037549.

Keena, M.A. and Moore, P.M. 2010. Effects of temperature on *Anoplophora glabripennis* (Coleoptera : Cerambycidae) larvae and pupae. Environmental Entomology, **39**: 1323–1335. https://doi.org/10.1603/En09369.

Klowden, M.J. 2013. Physiological systems in insects. Elsevier/Academic Press, Amsterdam, The Netherlands.

Koniger, A. and Grath, S. 2018. Transcriptome analysis reveals candidate genes for cold tolerance in *Drosophila ananassae*. Genes, **9**: article 624, 1–22. https://doi.org/10.3390/genes9120624.

Lawrence, J.F. 1991. Cerambycidae (Chrysomeloidea) (including Disteniidae, Hypocephalidae, Oxypeltidae, Parandridae, Spondylidae, Vesperiidae): long-horned beetles, round-headed wood borers. *In* Immature insects. *Edited by* F. W. Stehr. Kendall/Hunt Publishing, Dubuque, Iowa, United States of America. Pp. 556–561.

MacMillan, H.A., Knee, J.M., Dennis, A.B., Udaka, H., Marshall, K.E., Merritt, T.J.S., and Sinclair, B.J. 2016. Cold acclimation wholly reorganizes the *Drosophila melanogaster* transcriptome and metabolome. Scientific Reports, **6**: article 28999. https://doi.org/10.1038/srep28999.

MacMillan, H.A. and Sinclair, B.J. 2011. The role of the gut in insect chilling injury: cold-induced disruption of osmoregulation in the fall field cricket, *Gryllus pennsylvanicus*. Journal of Experimental Biology, **214**: 726–734. https://doi.org/10.1242/jeb.051540.

Monne, M.L., Monne, M.A., and Wang, Q. 2017. General morphology, classification, and biology of Cerambycidae. *In* Cerambycidae of the world: biology and pest management. *Edited by* Q. Wang. CRC Press, Boca Raton, Florida, United States of America. Pp. 2–66.

Nehme, M., Keena, M., Zhang, A.J., Sawyer, A., and Hoover, K. 2010. Monitoring Asian longhorned beetles in Massachusetts. *In* Proceedings, 21st U.S. Department of Agriculture interagency research forum on invasive species. *Edited by* K.A. McManus and K.W. Gottschalk. United States Department of Agriculture, Forest Service, Newtown Square, Pennsylvania, United States of America. Pp. 109–110.

Poupardin, R., Schottner, K., Korbelova, J., Provaznik, J., Dolezel, D., Pavlinic, D., *et al*. 2015. Early transcriptional events linked to induction of diapause revealed by RNA-seq in larvae of drosophilid fly, *Chymomyza costata*. BMC Genomics, **16**: article 720. https://doi.org/10.1186/s12864-015-1907-4.

Roe, A.D., Demidovich, M., and Dedes, J. 2018. Origins and history of laboratory insect stocks in a multispecies insect production facility, with the proposal of standardized nomenclature and designation of formal standard names. Journal of Insect Science, **18**: 1–9. https://doi.org/10.1093/jisesa/iey037.

Scully, E.D., Geib, S.M., Carlson, J.E., Tien, M., McKenna, D., and Hoover, K. 2014. Functional genomics and microbiome profiling of the Asian longhorned beetle (*Anoplophora*

glabripennis) reveal insights into the digestive physiology and nutritional ecology of wood feeding beetles. BMC Genomics, **15**: article 1096, 1–21. https://doi.org/10.1186/1471-2164-15-1096.

Sinha, R.N. 1958. The alimentary canal of the adult of *Tribolium castaneum* Herbst (Coleoptera, Tenebrionidae). Journal of the Kansas Entomological Society, **31**: 118–125. https://doi.org/10.1016/0020-7322(73)90014-7.

Svacha, P. and Lawrence, J.F. 2014. Cerambycidae Latreille. 1802. *In* Handbook of zoology - Arthropoda: Insecta. Coleoptera, beetles, morphology and systematics. *Edited by* R.A.B. Leschen and R.G. Beutel. Walter de Gruyter, Berlin, Germany. Pp. 77–177.

Teets, N.M., Peyton, J.T., Ragland, G.J., Colinet, H., Renault, D., Hahn, D.A., and Denlinger, D.L. 2012. Combined transcriptomic and metabolomic approach uncovers molecular mechanisms of cold tolerance in a temperate flesh fly. Physiological Genomics, **44**: 764–777. https://doi.org/10.1152/physiolgenomics.00042.2012.

Torson, A.S., Yocum, G.D., Rinehart, J.P., Nash, S.A., Kvidera, K.M., and Bowsher, J.H. 2017. Physiological responses to fluctuating temperatures are characterized by distinct transcriptional profiles in a solitary bee. Journal of Experimental Biology, **220**: 3372–3380. https://doi.org/10.1242/jeb.156695.

Wei, Y.D., Lee, K.S., Gui, Z.Z., Yoon, H.J., Kim, I., Je, Y.H., *et al*. 2006. N-linked glycosylation of a beetle (*Apriona germari*) cellulase Ag-EGase II is necessary for enzymatic activity. Insect Biochemistry and Molecular Biology, **36**: 435–441. https://doi.org/10.1016/j.ibmb.2006.03.007.

Figure Captions

Figure 1. External larval anatomy of *Anoplophora glabripennis*. **A**, Dorsal view of the head (h), pronotum, thoracic segments (t1–3), and abdominal segments (a1–10) **B**, lateral view.

Figure 2. Haemolymph collection from *Anoplophora glabripennis* larvae. **A**, A bleeding wound was created by puncturing the anterior edge of the prothoracic shield; **B**, fresh haemolymph (left) and haemolymph that has melanised following 15 minutes exposure to air.

Figure 3. Open body cavity. Larval morphology of the head, prothorax, fat body, and gut in *Anoplophora glabripennis*. **A**) Freeze killed; **B**, fresh killed.

Figure 4. Gut and associated structures. Foregut (blue), midgut (green), and hindgut (orange) of *Anoplophora glabripennis* larvae after removal of surrounding fat body. **A**, freeze killed; **B**, fresh killed.

Figure 5. Malpighian tubules and tracheae. Morphological differences between the Malpighian tubules and tracheae in both freeze-killed and fresh-killed *Anoplophora glabripennis* larvae. The Malpighian tubules emerge at the midgut/hindgut boundary, appear opaque (cloudy), and are repeated curved (bumpy). Tracheae appear hollow, shiny, and smooth in appearance. Tracheae and Malpighian tubules are similar in colour in freeze-killed specimens, but tubules are bright yellow in colour in fresh-killed larvae. Scale bars are 0.2 mm.

353 **Figure 6. Muscle.** Musculature for both freeze-killed and fresh-killed *Anoplophora glabripennis*
354 larvae. **A**, Head and thorax; **B**, abdomen.
355
356 **Figure 7.** Supraoesophageal ganglia. The supraoesophageal ganglia (brain) of *Anoplophora*
357 *glabripennis* larvae lies dorsal to the oesophagus and ventral to the prothoracic muscle. The
358 ganglion lobes are easily distinguished from surrounding tissue in both frozen and fresh-killed
359 specimens. **A**, Frozen specimens, **B**) fresh-killed specimens.
360