The light at the end of the tunnel: Photosensitivity in developing mountain pine beetle (*Dendroctonus ponderosae*)

by

Debra Lindsay Wertman

BSc (Honours, Co-op), University of Victoria, 2014
BFA (Honours), University of Victoria, 2008

A Thesis in Partial Fulfillment
of the Requirements for the Degree of

MASTER OF SCIENCE

in the Department of Biology

© Debra Lindsay Wertman, 2017

University of Victoria

All rights reserved. This thesis may not be reproduced in whole or in part, by photocopy
or other means, without the permission of the author.

SUPERVISORY COMMITTEE

The light at the end of the tunnel: Photosensitivity in developing mountain pine beetle (*Dendroctonus ponderosae*)

by

Debra Lindsay Wertman

BSc (Honours, Co-op), University of Victoria, 2014
BFA (Honours), University of Victoria, 2008

Supervisory Committee:

Dr. Steve Perlman, Department of Biology
Supervisor

Dr. Katherine Bleiker, Natural Resources Canada, Canadian Forest Service (Pacific Forestry Centre)
Outside Member

Dr. John Taylor, Department of Biology
Departmental Member

ABSTRACT

Supervisory Committee:

Dr. Steve Perlman, Department of Biology
Supervisor

Dr. Katherine Bleiker, Natural Resources Canada, Canadian Forest Service (Pacific Forestry Centre)
Outside Member

Dr. John Taylor, Department of Biology
Departmental Member

This research explores the capacity for functional photoreception in larvae of the mountain pine beetle (*Dendroctonus ponderosae*), an extremely important forest pest insect that is well adapted for development beneath the bark of pine trees. Phototaxis tests, gene expression analysis and development experiments were integrated to assess mountain pine beetle larvae for light sensitivity. When presented with a phototaxis choice test, larvae preferred dark over light microhabitats, revealing that larvae sense and respond behaviourally to light. Long wavelength opsin transcription was identified in all life stages, including eggs and larvae, suggesting that *D. ponderosae* possesses extraretinal photosensitive capabilities across its life cycle. The long wavelength opsin could function in phototaxis or the development phenology of immature beetles, while the ultraviolet opsin, only found to be expressed in pupae and adults, is likely to function in dispersal via the compound eyes. Results from two development experiments reveal an effect of photoperiod treatment on beetle development rate when reared from the egg stage, but not when reared from mature larvae, indicating that a critical photosensitive life stage(s) must occur in *D. ponderosae* prior to the third larval instar. An effect of photoperiod on adult emergence rates, however, appears to be independent of larval rearing conditions. The discovery of opsin expression and negative phototaxis in eyeless mountain pine beetle larvae, in addition to an effect of photoperiod on immature development and adult emergence rates, suggest that light and photoperiodism likely function in survival and life cycle coordination in this species.

TABLE OF CONTENTS

SUPERVISORY COMMITTEE ii

ABSTRACT iii

TABLE OF CONTENTS iv

LIST OF TABLES viii

LIST OF FIGURES xi

ACKNOWLEDGEMENTS xv

CHAPTER 1: General introduction 1

- **Insect photoperiodism** 1
- **Insect dormancy and diapause** 1
- **Insect circadian regulation (vs. photoperiodism)** 2
- **Photoperiodism and circadian regulation in cryptic insects** 3
- **Reception of photoperiodic information in insects** 5
- **Opsins** 6
- **Insect opsins** 7
- **Beetle opsins** 7
- **Insect photoreceptors (stemmata and extraocular)** 8
- **Extraretinal opsins** 10
- **Cryptochrome** 11
- **Gustatory-related receptor proteins** 11
- **Phototaxis** 12
- **Mountain pine beetle biology** 12

FRAMEWORK FOR STUDY 14

REFERENCES 15

CHAPTER 1 Figures 22

CHAPTER 2 23
Behavioural and genetic studies of photosensitivity in larvae of the mountain pine beetle (*Dendroctonus ponderosae*)

INTRODUCTION 23

Long wavelength light and low-light adaptations in insects 23

Ultraviolet light and navigation in insects 25

Bark beetle phototaxis 26

The mountain pine beetle 27

Framework for study 27

METHODS 30

Phototaxis experiment 30

Data analysis 33

Opsins – phylogenetic analysis 34

Identifying opsin sequences from the *D. ponderosae* genome 34

Opsin phylogenetic tree (amino acid) construction 35

Opsins – gene expression analysis 35

Primer design 36

RNA extraction and cDNA production 37

Standard curves 38

qPCR 38

Data analysis 40

RESULTS 42

Phototaxis experiment 42

Opsins – phylogenetic analysis 43

Opsins – gene expression analysis 44

Opsin expression across life stages 45

Long wavelength opsin expression in fourth instar larvae across photoperiods 45

Head versus body long wavelength opsin expression in fourth instar larvae 46

DISCUSSION 47

Phototaxis 47

Opsins – phylogeny and gene expression 49

Future directions 55

REFERENCES 57

CHAPTER 2 Tables 64

CHAPTER 2 Figures 66

CHAPTER 2 Appendix 75

CHAPTER 3 80
Effects of photoperiod on development and emergence rates in the mountain pine beetle (*Dendroctonus ponderosae*)

INTRODUCTION 80

Insect photoperiodism and circadian regulation 80

Insect dormancy 81

The mountain pine beetle 82

Dormancy in bark beetles 83

Framework for study 84

METHODS 86

Development experiment A 86

Photoperiod treatments 86

Insect source and sampling 87

Data analysis 88

Development experiment B 89

Photoperiod treatments 89

Insect source and sampling 89

Data analysis 90

Experimental treatment selection 91

RESULTS 93

Development experiment A 93

Development experiment B 93

DISCUSSION 95

Effect of photoperiod on development rate 95

Effect of photoperiod on emergence rate 97

Future directions 99

REFERENCES 102

CHAPTER 3 Tables 107

CHAPTER 3 Figures 108

CHAPTER 3 Appendix 113

LIST OF TABLES

CHAPTER 2 Tables 64

Table 1. Summary of primers designed for this study, including melt temperature, product size and nucleotide primer sequence 64

Table 2. Summary of *D. ponderosae* annotated opsin nucleotide and protein sequences (NCBI Resource Coordinators 2017) 64

Table 3. Summary of qPCR primer efficiencies, calculated from standard curves spanning six orders of magnitude, and melt curve peaks for the long wavelength opsin (LW), ultraviolet opsin (UV, no primer efficiency available), and reference (RPS3 and bAct) gene products 64

Table 4. Table summarizing the transcription status (+/- = present/absent, NA = not assessed) of the long wavelength and ultraviolet opsins across mountain pine beetle life stages [egg (n = 2), L1-4 = first to fourth instar larva, P = pupa, TA = teneral adult, EA = emerged adult (n = 4)] 65

CHAPTER 2 Appendix 75

Appendix Table 1. Samples from development experiments A and B (see CHAPTER 3) used for opsin gene expression analysis in this study (four sets of reactions: 1 = long wavelength opsin expression across photoperiods in larvae, 2 = head versus body long wavelength opsin expression in larvae, 3 = long wavelength opsin transcript presence/absence across life stages, 4 = ultraviolet opsin transcript presence/absence across life stages). L1-4 = first to fourth instar larva, P = pupa, TA = teneral adult, EA = emerged adult 75

Appendix Table 2. List of *Dendroctonus ponderosae, Tribolium castaneum, Apis mellifera, Drosophila melanogaster, Danio rerio and Homo sapiens* amino acid opsin sequences that were used to construct a phylogenetic tree 76

Appendix Table 3. Summary of *D. ponderosae* reference genes used for long wavelength opsin expression normalization in this study, and the homologous genes in *T. castaneum.* NCBI accession numbers (*T. castaneum* protein sequences, *D. ponderosae* whole-genome shotgun contig nucleotide sequences encoding reference proteins) and annotations included (* = identified by this study) 77

Appendix Table 4. ANOVA output table for the comparison of logR values in fourth instar *D. ponderosae* larvae (overall n = 16, n = 4 per treatment) across photoperiods (RPS3) 77

Appendix Table 5. ANOVA output table for the comparison of logR values in fourth instar *D. ponderosae* larvae (overall n = 16, n = 4 per treatment) across photoperiods (bAct) 77

Chapter2Appendix_Phototaxis.xlsx .....(attached), 78
Appendix Table 6. Phototaxis experiment data.
Appendix Table 7. Phototaxis control data.
Appendix Table 8. Phototaxis experiment temperature data.
Appendix Table 9. Phototaxis control temperature data.

Chapter2Appendix_qPCR.xlsx .....(attached), 78
Appendix Table 10. qPCR long wavelength opsin (LW) raw Cqs across life stages.
Appendix Table 11. qPCR reference gene (RPS3) raw Cqs across life stages.
Appendix Table 12. qPCR reference gene (bAct) raw Cqs across life stages.
Appendix Table 13. qPCR ultraviolet opsin (UV) raw Cqs across life stages.
Appendix Table 14. qPCR long wavelength opsin (LW) raw Cqs for L4s across photoperiods.
Appendix Table 15. qPCR reference gene (RPS3) raw Cqs for L4s across photoperiods.
Appendix Table 16. qPCR reference gene (bAct) raw Cqs for L4s across photoperiods.
Appendix Table 17. qPCR long wavelength opsin (LW) raw Cqs for L4s head/body.
Appendix Table 18. qPCR reference gene (RPS3) raw Cqs for L4s head/body.
Appendix Table 19. qPCR reference gene (bAct) raw Cqs for L4s head/body.

CHAPTER 3 Tables 107

Table 1. Results from the generalized linear models used to evaluate the effects of photoperiod on *D. ponderosae* development and emergence rates from experiment A (reared from field-collected third or fourth instar under photoperiod treatments in the laboratory). DD = degree day, Light = photoperiod treatment (0:24, 10:14, 17:7, 24:0), n = number of observations [number of samples used to calculate proportion (binomial), number emerged beetle degree day observations (Poisson)]. Significance of independent variables is denoted by an asterisk ($p < 0.05$) 107

Table 2. Results from the generalized linear models used to evaluate the effects of photoperiod on *D. ponderosae* development and emergence rates from experiment B (reared from eggs under photoperiod treatments in the laboratory). DD = degree day, Light = photoperiod treatment (0:24, 17:7), n = number of observations [number of samples used to calculate proportion (binomial), number of pupa/emerged beetle degree day observations (Poisson)]. Significance of independent variables is denoted by an asterisk ($p < 0.05$) 107

CHAPTER 3 Appendix 113

Appendix Table 1. Output of the experiment A binomial logistic model used to test for an effect of photoperiod (Light.h; 0:24, 10:14, 17:7, 24:0) and degree day (DD) on proportion *D. ponderosae* larvae sampled over time (significance level *** = p < 0.001) (RStudio Team 2015, R Core Team 2015) 113

Appendix Table 2. Output of the experiment A Poisson model used to test for an effect of photoperiod (Light.EA; 0:24, 10:14, 17:7, 24:0) on degree days to adult emergence (significance level *** = p < 0.001) in *D. ponderosae* (RStudio Team 2015, R Core Team 2015) 113

Appendix Table 3. Output of the experiment B binomial logistic model used to test for an effect of photoperiod (Light.h; 0:24, 17:7) and degree day (DD) on proportion *D. ponderosae* larvae sampled over time (significance level *** = p < 0.001, ** = p < 0.01) (RStudio Team 2015, R Core Team 2015) 113

Appendix Table 4. Output of the experiment A Poisson model used to test for an effect of photoperiod (Light.P; 0:24, 17:7) on degree days to pupation (significance level *** = p < 0.001) in *D. ponderosae* (RStudio Team 2015, R Core Team 2015) 114

Appendix Table 5. Output of the experiment B Poisson model used to test for an effect of photoperiod (Light.EA; 0:24, 17:7) on degree days to adult emergence (significance level *** = p < 0.001) in *D. ponderosae* (RStudio Team 2015, R Core Team 2015) 114

Chapter3Appendix_ExperimentA.xlsx .....(attached), 114
Appendix Table 6. Experiment A sample data.
Appendix Table 7. Experiment A emergence data.

Chapter3Appendix_ExperimentB.xlsx .....(attached), 114
Appendix Table 8. Experiment B sample and emergence data.

LIST OF FIGURES

CHAPTER 1 Figures 22

Figure 1. Occurrence map of mountain pine beetle in northwestern North America (adapted from Bleiker and Van Hezewijk 2016) 22

CHAPTER 2 Figures 66

Figure 1. SEM of a mountain pine beetle larva showing the lack of exterior photoreceptor organs (Wertman 2016) 66

Figure 2. SEM of an adult mountain pine beetle illustrating the apparent absence of ocelli (Wertman 2013) 66

Figure 3. Diagram illustrating the layering of phloem sandwich components, minus temperature probes, larvae, clips and elastic bands. Sandwiches were constructed from base and treatment glass panes, shore pine phloem squares (taupe), clear packing tape, black duct and masking tape (transparent black). Each finished unit exposed one half of each phloem punch to light (white) and the other to dark (black) 67

Figure 4. Photograph of a completed phloem sandwich containing temperature probes 68

Figure 5. Image showing the placement of a temperature probe within the phloem tissue 68

Figure 6. Phloem sandwiches (eight units = one run) arranged in a grid pattern within a white foam core frame, with a bank of two broad-spectrum fluroescent lights suspendend 33.5cm above to produce a maximum light intensity of 4740 lux 69

Figure 7. Light spectra profile for the broad-spectrum (6400K) fluorescent lights used for phototaxis experimentation, in mW/nm across wavelengths (nm) (SunBlaster Lighting 2016) 69

Figure 8. Diagram depicting the four different configurations of phloem sandwich units beneath the light panel (yellow outline) that were represented evenly across phototaxis experiment and control runs 70

Figure 9. Photograph of two experimental *D. ponderosae* larvae after dissection of a phloem sandwich. These larvae mined beneath the dark panel and were scored as having chosen dark. The dashed line shows the position of the light-dark panel interface in the intact unit 70

Figure 10. Counts of 158 insects by light preference across eight phototaxis experiment runs (light n = 57, dark n = 101). A chi-square analysis reveals a significant effect of light on *D. ponderosae* larval choice ($\chi^2_1 = 8.8327$: $p < 0.01$). An expected choice ratio was obtained from 71 control larvae assayed in the absence of light (34:37 L:D) 71

Figure 11. Amino acid phylogeny of *Dendroctonus ponderosae* visual opsins (long wavelength and ultraviolet-sensitive) aligned with *Tribolium castaneum, Apis mellifera, Drosophila melanogaster,* (select) *Danio rerio and Homo sapiens* Opn4 R-type (Provencio et al. 1998, 2000) and C-type opsin sequences. The Opn4 R-type opsin clade (lower) includes both insect visual opsins and vertebrate non-visual melanopsins, while the C-type opsin clade (above) includes vertebrate visual and insect non-visual opsins. Mountain pine beetle sequences are highlighted with an asterisk 72

Figure 12. Boxplot for comparison of fold-change long wavelength opsin expression (logR, RPS3) in fourth instar *D. ponderosae* larvae (overall n = 16, n = 4 per treatment) across photoperiods (ANOVA: $F_{3,12} = 0.826$: $p = 0.51$). Larvae were held for two weeks at treatment photoperiods prior to sampling and were collected during the photophase 73

Figure 13. Boxplot for comparison of fold-change long wavelength opsin expression (logR, bAct) in fourth instar *D. ponderosae* larvae (overall n = 16, n = 4 per treatment) across photoperiods (ANOVA: $F_{3,12} = 0.958$: $p = 0.44$). Larvae were held for two weeks at treatment photoperiods prior to sampling and were collected during the photophase 73

Figure 14. Boxplot of logR values for long wavelength opsin expression in fourth instar larval heads versus bodies (RPS3 reference gene) in larvae *D. ponderosae* (n = 7 larvae). Analysis reveals a significant difference in transcript level between body parts (paired t-test: $t_6 = 2.709$: $p < 0.05$), with expression lower in the body than the head 74

Figure 15. Boxplot of logR values for long wavelength opsin expression in fourth instar larval heads versus bodies (bAct reference gene) in larvae *D. ponderosae* (n = 7 larvae). Analysis reveals a significant difference in transcript level between body parts (paired t-test: $t_6 = 2.9126$: $p < 0.05$), indicating lower expression in the body than the head 74

CHAPTER 2 Appendix 75

Appendix Figure 1. Diagram illustrating the assembly order of phloem sandwich components, minus temperature probes, larvae, clips and elastic bands. 1) A phloem square was peeled away from the sapwood and placed phloem side up in the centre of a large pane of glass (15cm X 30cm) edged with black duct tape; 2) four 1.2cm X 0.5cm oval-shaped punches (for larvae) were made horizontally along the centre of the square; 3) the dark panel was secured to the sandwich using clear packing tape, ensuring even division of the punches; 4) black masking tape was placed along the outside edges of the phloem at the panel interface to block light from entering the dark treatment via the transparent pane; 5) the transparent panel was placed directly against the inside edge of the dark panel and attached with packing tape; 6) the entire sandwich was then secured tightly with packing tape, including all edges and along the panel interface; 7) black masking tape was added to the outer edge of the dark panel to seal the treatment from light; 8) (not shown) two elastics were placed lengthwise over the sandwich, followed by four 0.75" alligator clips (two per side) for tightening 79

CHAPTER 3 Figures 108

Figure 1. Wavelength output in W/nm for the F48T8/TL841/HO 4100K 44 Watt Hg 2L bulbs used to provide light treatment in development experiments A and B (Philips Lighting Holding B.V. 2017). The bulbs emit broad-spectrum light including wavelengths in the ultraviolet (UVA) range (320-400nm) 108

Figure 2. Plot of proportion larvae observed in response to degree day, separated by photoperiod, for experiment A (n = 24 observations across six time points). The fitted binomial model does not support an effect of photoperiod on *D. ponderosae* development rate ($z_{21} = 0.267$: $p = 0.79$). Dots represent proportion larvae at each sample time, while lines show the predicted model fit by photoperiod 109

Figure 3. Bar plot of emerged *D. ponderosae* adult counts by degree day, separated by photoperiod, for experiment A (overall n = 973 beetles; 0:24 n = 257, 10:14 n = 259, 17:7 n = 250, 24:0 n = 207). Trends indicate steeper emergence peaks at 0:24 and 10:14 (short day) photoperiods than at 17:7 (long day) and 24:0 photoperiods. There is a significant effect of photoperiod on number of degree days to emergence (Poisson model: $z_{971} = -6.682$: $p < 0.0001$) 110

Figure 4. Plot of proportion larvae in response to degree day, separated by photoperiod, for experiment B (n = 10 observations across five time points). Analysis reveals a significant effect of photoperiod on immature *D. ponderosae* development rate (binomial model: $z_7 = 2.637$: $p < 0.01$). Dots represent proportion larvae at each sample time, while lines show the predicted binomial model fit by photoperiod (0:24 or 17:7) 111

Figure 5. Bar plot displaying number of *D. ponderosae* pupae in response to degree day, separated by photoperiod, for experiment B (overall n = 69 pupae, 0:24 n = 32, 17:7 n = 37). Beetles appear to pupate earlier under constant darkness than at 17:7. There is a significant effect of photoperiod on degree days to pupation (Poisson model: $z_{67} = 16.85$: $p < 0.0001$) 111

Figure 6. Bar plot of the number of emerged *D. ponderosae* adults in response to degree day, separated by photoperiod, from experiment B (overall n = 310 beetles; 0:24 n = 114, 17:7 n = 175). The beetles exhibit a steeper emergence peak in constant darkness than under long day (17:7) conditions. Analysis reveals a significant effect of photoperiod on number of degree days to emergence (Poisson model: $z_{308} = 20.08$: $p < 0.0001$) 112

ACKNOWLEDGMENTS

This research was graciously facilitated by my co-supervisors Dr. Katherine Bleiker (Natural Resources Canada, Canadian Forest Service, Pacific Forestry Centre) and Dr. Steve Perlman (University of Victoria), in addition to my committee member Dr. John Taylor (UVic). I would like to thank Greg Smith (Bleiker lab, NRCan), Dr. Matthew Ballinger (Perlman lab, UVic), Dr. Brian Van Hezewijk (NRCan), Ross Benton (NRCan), and various other members of the Perlman lab (UVic) for valuable insight during this project. Funding was provided by SERG International, Forest Protection Limited, and the Provinces of Alberta, Saskatchewan, Manitoba and Ontario. I am extremely grateful for the supportive working relationship among myself, the Government of Canada (Natural Resources Canada, Canadian Forest Service, Pacific Forestry Centre), and the University of Victoria. I also extend my gratitude to current and former co-op student in the Bleiker lab for their assistance with my project, including Maliya Cassels, Eleanor Stewart, Chelsea Dunbar, Erin McLeod. Finally, I would like to thank my incredibly supportive family, including my husband Todd Lambeth, parents Sam and Cheryl Wertman, sister Jaime Wertman (a fellow scientist and consistent source of moral support) and my miniature schnauzer, Wayne.

CHAPTER 1:

General introduction

Insect photoperiodism

The earth's rotation around its axis produces daily light/dark cycles and the rotation of earth around the sun results in seasonal shifts in day lengths, which become increasingly divergent by season proceeding polewards from the equator (Bradshaw and Holzapfel 2007). Photoperiodism is the mechanism by which organisms are able to anticipate seasonal events using photoperiod (day length) as a cue, regulating current physiological and developmental mechanisms that are important to the insects life cycle in future time or space. Life cycle timing is essential to survival and fitness in seasonal environments, and photoperiod provides insects with a consistent and predictable signal of future environmental shifts. Insects are generally able to synchronize development and life cycles by using photoperiodic time measurement, responding developmentally or behaviourally to circadian oscillations. Photoperiodism may play a role in migration, dormancy, reproductive and developmental programming, sometimes triggering an "irrevocable cascade" of events.

Insect dormancy and diapause

A common photoperiodic response in insects is the induction of diapause, a form of dormancy, which results from the perception of shortening day lengths in many species (Bradshaw and Holzapfel 2001). The diapause process is characterized by programmed metabolic suppression and developmental arrest in response to environmental thresholds, and is thought to involve hormonal and clock gene regulation (Koštál 2006, Belozerov 2009). Initiated prior to the onset of adverse conditions, diapause is induced when the insect receives token stimuli that signal seasonal change, and is terminated in response to specific cues. Diapause may be obligate, genetically-determined for a specific life stage, or facultative, triggered by environmental factors (Belozerov 2009). The life stage that is sensitive to diapause inducing stimuli occurs prior to the onset of adverse conditions, and sometimes in the previous generation (Hut et al. 2013).

Quiescence, a type of non-diapause dormancy, may be defined as the immediate decline and resumption of developmental processes in response to environmental thresholds (Koštál 2006, Belozerov 2009). While quiescence is typically not implicated in life cycle synchronicity, stage-specific quiescence may act to synchronize annual life cycles (Belozerov 2009). Cold induced quiescence, facilitating survival during exposure to low temperatures, is often found in temperate insects. The precise physiological differences between diapause and non-diapause dormancy are largely unresolved, as is the role of non-diapause dormancy in life cycle regulation and synchronicity.

Seasonality is increasingly more pronounced in the environment and influential on insect phenology as latitude increases towards the poles (Bradshaw and Holzapfel 2007). The critical photoperiod, the day length at which dormancy is induced in 50% of an insect population, typically increases with latitude and altitude (Danileveskii 1965, Bradshaw and Holzapfel 2007). Steeper effects of photoperiod on insect phenology are observed at high latitudes, with increased response (diapause incidence, development rate) to longer photoperiods that are coincident with the earlier arrival of fall in these regions (Bradshaw and Holzapfel 2001, Hut et al. 2013). The pitcher-plant mosquito *Wyeomyia smithii* exhibits a photoperiodically induced larval diapause, for which the critical photoperiod is genetically determined and varies with latitude and altitude (Bradshaw and Holzapfel 2001, Emerson et al. 2009). Interestingly, the critical photoperiod for *W. smithii* diapause induction has shifted in response to climate change across its range, with the strongest effects leading to shortening critical photoperiods observed in northern populations (Bradshaw and Holzapfel 2001). This shift equates to the critical photoperiod arriving nine days later in 1996 than 1972 (a decrease of 0.6 hours) (Bradshaw and Holzapfel 2001), and has likely shifted more between 2001 and the publication of this thesis (2017).

Insect circadian regulation (vs. photoperiodism)

The circadian clock consists of an endogenous light-independent physiological rhythm that can be entrained to the daily light-dark cycle via extraretinal light perception, while the photoperiodic clock is responsible for interpreting and accumulating day lengths for future physiological programming (Bradshaw and Holzapfel 2010). Light-dark

periodicity generates zeitgeber stimuli, which function as entrainment cues for the circadian clock (Fleissner et al. 1993, Helfrich-Förster et al. 2001, Komada et al. 2015). Circadian rhythms are expressed under constant conditions and can be reset (or phase-shifted) by an interruption of constant stimuli (Bradshaw and Holzapfel 2010). In contrast, the photoperiodic clock does not depend on entrainment by a 24 hour light-dark cycle, and instead functions as an interval timer. Rhythmicity is not an aspect of the insect photoperiodic response, a threshold-initiated physiological event cascade (Bradshaw and Holzapfel 2010), whereas rhythmic behaviours under circadian control may include eclosion, oviposition, egg hatching, flight and movement (Felisberti et al. 1997, Lampel et al. 2005). A molecular link between insect photoperiodism and circadian regulation is supported by recent literature, for example the demonstration of a functional association between clock genes and photoperiodism in *Drosophila* (Sandrelli et al. 2007, Tauber et al. 2007) and the bean bug *Riptortus pedestris* (Ikeno et al. 2010), although the two clocks may have evolved independently of one another in *W. smithii* (Emerson et al. 2009).

Photoperiodism and circadian regulation in cryptic insects

Circumstantial evidence of photoperiodism and circadian rhythmicity in cryptic (unexposed) insects is apparent in the literature, for species discussed throughout CHAPTER 3 [immature European spruce bark beetle *Ips typographus* (Curculionidae, Scolytinae) (Doležal and Sehnal 2007), yellow-spotted longicorn beetle *Psacothea hilaris* (Shintani et al. 1996) and chrysanthemum longicorn beetle *Phytoecia rufiventris* (Shintani 2011) (Cerambycidae)], as well as for insects presented in this section, the cave beetle *Laemostenus latialis* (Carabidae) (Pasquali and Sbordoni 2014), diapausing adult Colorado potato beetle (*Leptinotarsa decemlineata* (Chrysomelidae) (Lefevere and de Kort 1989), and periodical cicada nymphs (*Magicicada* spp.) (Heath 1968, Wolda 1989, Williams and Simon 1995, Karban et al. 2000). While troglomorphic organisms exhibit arrhythmic periodicity in behaviours, thought to represent complete adaptation to subterranean conditions through the degeneration of circadian rhythmicity (Pasquali and Sbordoni 2014, Stringer and Meyer-Rochow 2017), researchers identified rhythmic

locomotory patterns amongst a population of *L. latialis* that were synchronous with the circadian movements of surface beetles (Pasquali and Sbordoni 2014).

Adult diapause in *L. decemlineata* occurs deep in the soil (maximum 60cm) and is both induced and maintained by short day length (de Wilde 1955, Lefevere and de Kort 1989). *L. decemlineata* diapause is followed by a period of diapause development, and despite their depth in the soil during this time, the insects retain photoperiodic sensitivity to diapause inducing cues and are unresponsive to temperature fluctuations (Lefevere and de Kort 1989, de Kort 1990). Diapausing *L. decemlineata* females that were removed from the substrate and exposed to experimental photoperiods required at least three long day cycles for termination of diapause development, and that exposure to short day photoperiods negatively influenced oviposition (Lefevere and de Kort 1989).

Thirteen- and 17-year periodical cicadas (*Magicicada* spp.) could potentially provide a fascinating system for the study of photoperiodism in cryptic insect species. Cicada nymphs develop underground, feeding on root xylem sap for 13 or 17 years, until entire cohorts (sometimes in the millions) emerge from the soil synchronously within days or weeks of one another (Heath 1968, Williams and Simon 1995, Karban et al. 2000). Nymphs from a given cohort exhibit different growth rates, and reside at depths of 15-60cm throughout their development (Karban et al. 2000). The environmental stimuli functioning as seasonal cues for cicada activity must be highly regular and reliable (Wolda 1989), as periodical cicadas are rarely observed emerging one year early or late (Heath 1968, Williams and Simon 1995). Although soil temperature may fluctuate on a daily and annual basis at the depths and latitudes at which the nymphs are found, this fluctuation can be highly variable and may be ineffective in generating the predictable stimuli required for seasonal time measurement (Wolda 1989, Williams and Simon 1995, Karban et al. 2000), although a critical soil temperature may contribute to emergence timing (Heath 1968, Williams and Simon 1995). In the tropical Panamanian cicada *Stenotarsus rotundus*, Wolda (1989) observed no relationship between emergence and wet/dry seasonal cues. Cicada nymphs are apparently equipped with mechanisms for seasonal counting and emergence synchronicity (Heath 1968, Karban et al. 2000), and I

suggest that the insects may use low-light sensitive photoreceptors to perceive seasonal shifts in day length from within the soil.

Other time measurement strategies that have been proposed for the periodical cicada include the accumulation of winter diapause counts (Heath 1968) and the seasonal tracking of xylem composition (Wolda 1989, Williams and Simon 1995, Karban et al. 2000). In a laboratory experiment, Karban et al. (2000) applied a six month photo- and thermoperiodic seasonal cycle to plants hosting 17-year periodical cicadas, collected from the field in their 15th year, and successfully induced emergence one year early. The authors hypothesize that the cicadas counted seasonal host cycles and suggest that the insects likely did not perceive experimental photoperiod or thermoperiod directly from within the soil.

Reception of photoperiodic information in insects

The physiological photoperiodic response involves photoreception, day length measurement and accumulation of effects leading to endocrine-initiated developmental events (Saunders and Bertossa 2011). Photoreceptors such as compound eyes, ocelli and stemmata are not necessarily involved in the photoperiodic response, as extraretinal photoreception of zeitgeber stimuli is often situated in the brain (Numata et al. 1997, Saunders 2012). Extraretinal photoreceptors in the insect brain have been demonstrated to function independently of retinal receptors in circadian photoreception in many species, although compound eyes and stemmata may also be the primary receptors of photoperiodic information in other species (Saunders 2012). It is hypothesized that holometabolous insects use opsin and cryptochrome facilitated extraocular photoreception (see CHAPTER 1: Extraretinal opsins, Cryptochrome) for circadian entrainment because the insects lack compound eyes as larvae, while non-holometabolous insects including crickets and cockroaches may rely solely on the compound eyes for the reception of photoperiodic stimuli (Komada et al. 2015).

While photoreceptors involved in the reception of zeitgeber stimuli in insects are diverse, including compound eyes, stemmata, cryptochrome proteins, and opsins expressed in the

brain, processing of photoperiodic information in all cases involves the optic lobes (Saunders 2012). Extraretinal photoperiodic receptors in insects are most sensitive to blue and green light (Numata et al. 1997); Saunders (2012) has proposed that opsins sensitive to longer wavelengths may be more directly involved in developmental regulation (diapause, for example), while blue light sensitive photoreceptors may be primarily involved in direct response to photoperiodic oscillation. Helfrich-Förster et al. (2001) demonstrated that at least three photoreceptors function in *Drosophila* circadian entrainment, cryptochrome, the compound eyes, and extraocular receptors (possibly the Hofbauer-Buchner eyelet).

Opsins

Opsins, the photoreceptive proteins facilitating all animal vision, belong to the G protein-coupled receptor (GPCR) superfamily (Porter et al. 2012). The proteins consist of seven transmembrane helices with a lysine in the seventh helix for light-sensitive chromophore molecule binding. Opsins function in both vision and non-visual light detection, and with a common ancestry, are highly conserved in structure among all animals. There are multiple classes of opsins found in animals, including the vertebrate and invertebrate visual and non-visual C-type (ciliary) and R-type (rhabdomeric) opsins, which may be expressed in image-forming photoreceptors and non-retinal tissue. While both R-type and C-type opsins may have extraretinal function in invertebrates, arthropod vision is enabled by R-type opsins (Porter 2016).

A visual pigment is formed by an opsin bound to a chromophore, which is usually derived from vitamin A (Wald 1968). Phototransduction begins when visual pigments absorb photons. The covalent interaction between the opsin and the chromophore enables light detection and determines the spectral sensitivity of the pigment (Porter et al. 2012). Colour discrimination emerges from the ability to compare input from multiple opsins with different spectral sensitivities (Martin et al. 2015), particularly via trichromatic (or greater) vision (Svaetichin and MacNichol 1958). The sensitivity of an opsin to certain wavelengths can be re-configured through amino acid sequence alterations or changes in chromophore structure. Ramirez et al. (2016) determined that

the common ancestor of all bilaterians had at least nine opsin paralogs in its genome, including R-type and C-type opsins, which likely were initially expressed in extraretinal cells and eventually incorporated into retinal photoreceptor organs during their evolution.

Insect opsins

The ancestral condition in insects is thought to be trichromatic vision with single copies of ultraviolet (300-400nm, peak 350nm), blue (400-500nm, 440nm), and long wavelength (480-600nm, 530nm) sensitive opsins (Jackowska et al. 2007, Martin et al. 2015, Lord et al. 2016, Sharkey et al. 2017). Insect visual opsins belong to the Opn4 R-type opsin subfamily, which also contains the vertebrate melanopsins (Provencio et al. 1998, 2000). Spectral response curves of opsins have distinctive peak sensitivities and tails that may overlap one another (Porter et al. 2012). Differentiation of opsin paralogs in ommatidia (compound eye units) photoreceptors is necessary for colour discrimination (Jackowska et al. 2007). While there are seven opsins known in *Drosophila* (Richards et al. 2008), and eleven in *Anopheles gambiae* (Jenkins and Muskavitch 2015), opsins have undergone considerably more duplications in highly visual adult dragonflies (Futahashi et al. 2015). Futahashi et al. (2015) identified more than 20 distinct opsin genes in one family of dragonflies (Libellulidae), and a total of 15-33 opsins, including 11-30 visual, across ten Odonate families.

Beetle opsins

The blue insect opsin was lost in the Neuropteroidea ancestor before the emergence of beetles, approximately 300 million years ago (Sharkey et al. 2017). As confirmation of this evolutionary loss of gene function, Sharkey et al. (2017) examined transcriptomes spanning 29 beetle families and failed to recover blue opsin transcripts. The authors conclude that the Neuropteroidea ancestor in which the loss occurred was likely nocturnal or low light adapted, and therefore not reliant on trichomacy for colour vision. Although all beetles lack a blue sensitive opsin, this is inconsistent with blue spectral sensitivities noted in some beetles including buprestids, chrysomelids, coccinellids, carabids and lampyrids (Lord et al. 2016, Sharkey et al. 2017).

Apparently, independent ultraviolet and long wavelength opsin duplications and spectral tuning events have contributed to the re-establishment of trichromatic vision and blue light sensitivity in many extant beetles (Lord et al. 2016). The emerald ash borer, *Agrilus planipennis* (Buprestidae), expresses two ultraviolet and two long wavelength opsins, with a third long wavelength opsin that is thought to be a larva-specific copy. The pollen beetle *Brassicogethes aeneus* has three ultraviolet opsin and two long wavelength opsin copies in its repertoire, evidence that beetle pollinators have secondarily acquired trichromacy as well (Sharkey et al. 2017). Most beetles, however, possess single ultraviolet and long wavelength opsin copies; beetles without opsin duplications that are reported to have blue sensitivity (fireflies, for example) may rely on structural screening filtering of light instead of spectral tuning (Martin et al. 2015, Lord et al. 2016).

Insect photoreceptors (stemmata and extraocular)

Stemmata are defined as the simple eyes of holometabolous insect larvae, not to be confused with the ocelli of adult holometabolous insects and non-holometabolous larvae (Gilbert 1994). Stemmata appear to derive from the compound eyes of non-holometabolous insects from the fusion of ommatidia, individual photoreceptive cell units within the retina (Liu and Friedrich 2004, Stecher et al. 2016). Six pairs of stemmata is thought to be the ancestral condition in beetle larvae (Gilbert 1989). In *Tribolium castaneum*, larval stemmata emerge from the posterior margin of the eye lobes in the embryonic stage, and are each attached via a single optic nerve deep in the brain (Liu and Friedrich 2004). In *Drosophila*, which exhibit derived photoreception machinery compared to Coleoptera, Bolwig organs function as internal eye in larvae, and the adult Hofbauer-Buchner eyelet is homologous to the internalized stemmata observed in adult beetles (discussed below) (Liu and Friedrich 2004, Lampel et al. 2005).

Vision in most holometabolous insects is simplified, facilitated by pairs of stemmata (Gilbert 1989). Stemmata are most highly developed in a few species of predaceous beetle larvae, including tiger beetles (Cicindelidae) larvae (Gilbert 1994, Gilbert 1989). Tiger beetle larvae are sit and wait predators that rely on relatively complex vision for movement detection, functioning in prey capture and eliciting a predator escape response

(retreat into burrow) (Gilbert 1989). Likewise, the complex stemmata of the sunburst diving beetle, *Thermonectus marmoratus* (Dytiscidae), assist the aquatic predatory larvae in prey capture (Stecher et al. 2016).

In holometabolous insects, larval stemmata migrate into the brain during metamorphosis and are retained inside the optic lobe, functioning as internal extraocular photoreceptors (Gilbert 1994, Felisberti et al. 1997). This phenomenon is thought to be ubiquitous among beetles, and cerebral extraocular photoreceptors have been identified in numerous species including *Tenebrio molitor* (Tenebrionidae), *Hylotrupes bajulus* (Cerambycidae), *Anthia sexguttata* (Carabidae), *Epilachna varivestis* and *Adalia bipunctata* (Coccinellidae) (Schulz et al. 1984, Fleissner et al. 1993, Felisberti et al. 1997). The photoreceptive cell clusters originating from stemmata can be observed macroscopically as dark spots between the medulla and the lamina in the anterior of the optic lobe, while rhabdomere-like structures and screening pigments (stemmata components) can be viewed at higher magnification (Felisberti et al. 1997). Internalized adult stemmata exhibit broad sensitivity to green light (524nm), which corresponds to peaks observed for larval stemmata. Cerebral extraocular photoreceptors and adult stemmata are implicated in the reception of photoperiodic stimuli in several insect orders (Lampel et al. 2005).

Two interesting extraocular photoreceptor organs were identified in the adult optic lobes of a carabid beetle, *Pachymorpha sexguttata*, that are unrelated to internal adult stemmata (Fleissner et al. 1993). The first organ is located at the fronto-dorsal lamina rim beneath the neurolemma and connects to the accessory medulla, while the second organ is located between the lobula and medulla. The well-organized tissue structure of this organ in pupae suggests that it may serve an important developmental function. Fleissner et al. (1993) implicated these extraocular photoreceptors in *P. sexguttata* circadian entrainment by identifying clock gene (period) expression within the organs. Spaethe and Briscoe (2005) have speculated that the lobula and lamina organs of the optic lobe may function in extraocular photoreception in both holometabolous and non-holometabolous insects.

Extraretinal opsins

At least six of ten photoreceptive protein classes currently known to exist across biological systems, including opsins, cryptochromes, and gustatory-related receptor proteins, may be expressed extraretinally in animals (Porter 2016). All ancestral opsins are thought to have been extraocular in function, and thus insect visual opsins must have evolved from extraretinal orthologs. Opsin transcripts and proteins have been detected in many extraretinal animal tissues, and are considered to have retained or re-evolved functions unrelated to image formation (Porter et al. 2012). Discoveries of opsin expression in such a diversity of non-retinal cells and tissues in vertebrates and invertebrates suggests that opsins may have pleiotropic functions, both light-dependent and independent. For example, visual and non-visual opsins expressed in the insect brain may be involved in the reception of photoperiodic information and the regulation of endogenous clocks (Velarde et al. 2005, Tierney et al. 2015).

Evidence of opsin expression in insect cerebral cells and tissues is widely reported (Shimizu et al. 2001, Lampel et al. 2005, Spaethe and Briscoe 2005, Velarde et al. 2005). Boceropsin is a green sensitive (long wavelength, 500-550nm) cerebral opsin expressed in larval silkworm (*Bombyx mori*) extraocular brain cells (Shimizu et al. 2001). Lampel et al. (2005) identified a cerebral green sensitive opsin expressed in the optic lobes and associated neurons of four species of hawkmoths (Sphingidae), which is not expressed in either the larval stemmata or adult compound eyes. The honey bee (*Apis mellifera*) expresses a C-type opsin called pteropsin in the adult brain that is not found in the compound eyes or ocelli (Velarde et al. 2005). Ultraviolet opsin expression in the bumblebee (*Bombus impatiens*) occurs in the retina, ocelli and in the optic lobes (Spaethe and Briscoe 2005). All of these opsins have been speculated to function in photoperiodism and/or circadian regulation (Shimizu et al. 2001, Lampel et al. 2005, Spaethe and Briscoe 2005, Velarde et al. 2005).

Cryptochrome

Cryptochrome proteins are involved in circadian oscillations of all metazoans (Yuan et al. 2007). In *Drosophila melanogaster*, the circadian regulation is a function of a transcriptional feedback loop involving a number of clock genes and their encoding proteins, including cryptochrome 1 (Saunders 2012). While cryptochrome 1 is known to be a blue light photoreceptor, cryptochrome 2 is thought to be photo-insensitive and function in light independent clock regulation (Yuan et al. 2007). Cryptochrome 1 is not present in *Apis mellifera, Tribolium castaneum* (Yuan et al. 2007), *Ptomaphagus hirtus* (Friedrich et al. 2011), nor *Dendroctonus ponderosae* (Keeling et al. 2013, NCBI Resource Coordinators 2017), which each appear to possess only cryptochrome 2.

Gustatory-related receptor proteins

Another class of photoreceptors worth discussing in the context of insect extraretinal reception are the gustatory-related receptor proteins (Porter 2016). These proteins are 7 transmembrane domain gustatory receptors for which light sensitivity has been reported in larval *Drosophila melanogaster* (Xiang et al. 2010) and *Caenorhabditis elegans* (Gong et al. 2016). These proteins are thought to be G-protein independent, possibly associated with chromophore, and activated by short wavelength light (Porter 2016). Another interesting feature of the gustatory-related receptor proteins is the inverted membrane topology (extracellular C terminus, intracellular N terminus) compared to other GPCRs, including opsins (extracellular N terminus, intracellular C terminus).

Drosophila melanogaster larvae have class IV dendritic arborization neurons lining the body wall, which exhibit sensitivity to ultraviolet, violet and blue light, especially at high intensities (Xiang et al. 2010). This response is enabled by the gustatory-related receptor protein Gr28b (determined via gene knockdown), which is the homolog of the photosensitive *Caenorhabditis elegans* protein LITE-1 that is similarly responsive to ultraviolet light (Gong et al. 2016). In *C. elegans,* LITE-1 is expressed in photosensitive neurons and is highly efficient at photon capture, determined from the absorbance properties of the purified protein (measured via spectrophotometry).

Phototaxis

The tropism theory by Jacques Loeb states that bilateral organisms can gather information about their surroundings through the perception of symmetric or asymmetric stimuli to either side of the body (Loeb 1912, Kane et al. 2013). Phototaxis, a type of tropism defined as movement in response to a gradient of light, may exhibit either positive or negative directionality (Chen et al. 2012). Phototaxis directionality may change throughout development, or in response to temperature, olfactory or moisture cues, to facilitate food location and dispersal (Gilbert 1994, Jackowska et al. 2007, Chen et al. 2012). Functions of negative phototaxis may include predator avoidance and evasion of damaging sunlight exposure (Edwards et al. 2008, Xiang et al. 2010).

Beetles exhibit a range of phototactic behaviours depending on habitat and life stage (Shepherd 1966, Safranyik et al. 1989, Gilbert 1994, Jackowska et al. 2007). All postembryonic stages of *Tribolium castaneum* are negatively phototactic, which likely reflects long term adaptation to a cryptozoic habit (Jackowska et al. 2007). The mealworm beetle (*Tenebrio molitor*) is generally negatively phototactic but becomes positively phototactic if desiccated. The Colorado potato beetle (*Leptinotarsa decemlineata*) exhibits positive phototaxis in the larval stage, presumably to increase encounter rates with suitable food plant tissue (Gilbert 1994).

Mountain pine beetle biology

The mountain pine beetle *Dendroctonus ponderosae* Hopkins (Coleoptera: Curculionidae, Scolytinae) is the most significant pest of mature pine forests in western North America (Safranyik and Carroll 2006, Safranyik et al. 2010), and has understandably been the subject of intensive ecological research for decades. The beetle is native to British Columbia, though historically populations were stable at endemic levels (Safranyik and Carroll 2006). The current epidemic erupted in the early 1990s and is spreading across the boreal forest and beyond its historic range into northern British Columbia, and east to north central Alberta (Safranyik et al. 2010) (Figure 1.). *D. ponderosae* population dynamics are influenced by both density dependent and

independent (climate) factors across its range (Bentz et al. 2013). Mountain pine beetle usually complete their life cycle in one year (univoltine), although a single generation may require two years to complete development (semivoltine) under cool conditions at high elevation or latitude (Amman 1973). *D. ponderosae* does not have an obligate winter diapause, but does possess temperature thresholds that function to synchronize development and adult emergence to achieve effective mass attack densities (Safranyik and Carroll 2006).

FRAMEWORK FOR STUDY

Mountain pine beetle development occurs entirely within the phloem layer of the host tree, from which the adult beetles emerge and disperse. I am interested in learning whether these insects interact with light from beneath the bark, and if so, uncovering possible functions of light perception in *D. ponderosae* larvae. For my MSc research, I integrated a variety of methods to assess larvae for evidence of photosensitivity, including larval phototaxis tests, gene expression analysis and development experiments, while carefully controlling for the confounding effects of temperature. I suggest that light sensitive opsin proteins, the molecules that enable all animal vision (Porter et al. 2012), may facilitate extraretinal photoreception in mountain pine beetle larvae. Photosensitivity in larvae could function in phototaxis or the perception of day length for circadian regulation or downstream physiological events. It may be circumstantially supported that the under-bark environment is not entirely aphotic, and that the beetles are consistently exposed to low levels of light.

REFERENCES

Amman, G. D. 1973. Population changes of the mountain pine beetle in relation to elevation. Environmental Entomology 4:541–547.

Belozerov, V. N. 2009. New aspects in investigations of diapause and non-diapause dormancy types in insects and other arthropods. Entomological Review 89:127–136.

Bentz, B., J. Vandygriff, C. Jensen, T. Coleman, P. Maloney, S. Smith, A. Grady, and G. Schen-langenheim. 2013. Mountain pine beetle voltinism and life history characteristics across latitudinal and elevational gradients in the western United States. Forest Science 60:434–449.

Bleiker, K. P. and B. H. Van Hezewijk. 2016. Flight period of mountain pine beetle (Coleoptera: Curculionidae) in its recently expanded range. Environmental Entomology 45: 1561-1567.

Bradshaw, W. E., and C. M. Holzapfel. 2001. Genetic shift in photoperiodic response correlated with global warming. Proceedings of the National Academy of Sciences of the United States of America 98:14509–14511.

Bradshaw, W. E., and C. M. Holzapfel. 2007. Evolution of Animal Photoperiodism. Annual Review of Ecology, Evolution, and Systematics 38:1–25.

Bradshaw, W. E., and C. M. Holzapfel. 2010. What Season Is It Anyway? Circadian Tracking vs. Photoperiodic Anticipation in Insects. Journal of Biological Rhythms 25:155–165.

Chen, Y., C. W. Luo, R. P. Kuang, H. W. Li, Z. Chen, and Y. J. Liu. 2012. Phototactic behavior of the Armand pine bark weevil, *Pissodes punctatus*. Journal of Insect Science 13:1–10.

Danilevskii A. S. 1965. Photoperiodism and seasonal development in insects. Oliver & Boyd Ltd., London, UK.

Doležal, P., and F. Sehnal. 2007. Effects of photoperiod and temperature on the development and diapause of the bark beetle *Ips typographus*. Journal of Applied Entomology 131:165–173.

Edwards, S. L., N. K. Charlie, M. C. Milfort, B. S. Brown, C. N. Gravlin, J. E. Knecht, and K. G. Miller. 2008. A novel molecular solution for ultraviolet light detection in *Caenorhabditis elegans*. PLoS Biology 6:1715–1729.

Emerson, K. J., S. J. Dake, W. E. Bradshaw, and C. M. Holzapfel. 2009. Evolution of photoperiodic time measurement is independent of the circadian clock in the pitcher-plant mosquito, *Wyeomyia smithii*. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology 195:385–391.

Felisberti, F., D. F. Ventura, and H. Hertel. 1997. Cerebral extraocular photoreceptors in beetles. Comparative Biochemistry and Physiology 118A:1353–1357.

Fleissner, G., G. Fleissner, and B. Frisch. 1993. A new type of putative non-visual photoreceptors in the optic lobe of beetles. Cell and Tissue Research 273:435–445.

Friedrich, M., R. Chen, B. Daines, R. Bao, J. Caravas, P. K. Rai, M. Zagmajster, and S. B. Peck. 2011. Phototransduction and clock gene expression in the troglobiont beetle *Ptomaphagus hirtus* of Mammoth cave. Journal of Experimental Biology 214:3532–3541.

Futahashi, R., R. Kawahara-Miki, M. Kinoshita, K. Yoshitake, S. Yajima, K. Arikawa, and T. Fukatsu. 2015. Extraordinary diversity of visual opsin genes in dragonflies. Proceedings of the National Academy of Sciences of the United States of America 112:E1247–E1256.

Gilbert, C. 1989. Visual determinants of escape in tiger beetle larvae (Cicindelidae). Journal of Insect Behavior 2:557–574.

Gilbert, C. 1994. Form and function of stemmata in larvae of holometabolous insects. Annual Review of Entomology 39:323–349.

Gong, J., Y. Yuan, A. Ward, L. Kang, B. Zhang, Z. Wu, J. Peng, Z. Feng, J. Liu, and X. Z. S. Xu. 2016. The *C. elegans* taste receptor homolog LITE-1 is a photoreceptor. Cell 167:1252–1263.

Heath, J. E. 1968. Thermal synchronization of emergence in periodical “ 17-year ” cicadas (Homoptera, Cicadidae, *Magicicada*). The American Midland Naturalist 80:440–448.

Helfrich-Förster, C., C. Winter, A. Hofbauer, J. C. Hall, and R. Stanewsky. 2001. The circadian clock of fruit flies is blind after elimination of all known photoreceptors. Neuron 30:249–261.

Hut, R. A., S. Paolucci, R. Dor, C. P. Kyriacou, and S. Daan. 2013. Latitudinal clines: an evolutionary view on biological rhythms. Proceedings of the Royal Society B: Biological Sciences 280:1–9.

Ikeno, T., S. I. Tanaka, H. Numata, and S. G. Goto. 2010. Photoperiodic diapause under the control of circadian clock genes in an insect. BMC Biology 8:116.

Jackowska, M., R. Bao, Z. Liu, E. C. McDonald, T. A. Cook, and M. Friedrich. 2007. Genomic and gene regulatory signatures of cryptozoic adaptation: Loss of blue sensitive photoreceptors through expansion of long wavelength-opsin expression in the red flour beetle *Tribolium castaneum*. Frontiers in Zoology 4:1–11.

Jenkins, A. M., and M. A. T. Muskavitch. 2015. Crepuscular behavioral variation and profiling of opsin genes in *Anopheles gambiae* and *Anopheles stephensi* (Diptera: Culicidae). Journal of Medical Entomology 52:296–307.

Kane, E. a, M. Gershow, B. Afonso, I. Larderet, M. Klein, A. R. Carter, B. L. de Bivort, S. G. Sprecher, and A. D. T. Samuel. 2013. Sensorimotor structure of *Drosophila* larva phototaxis. Proceedings of the National Academy of Sciences of the United States of America 110:E3868–E3877.

Karban, R., C. A. Black, and S. A. Weinbaum. 2000. How 17-year cicadas keep track of time. Ecology Letters 3:253–256.

Keeling, C. I., M. M. S. Yuen, N. Y. Liao, T. Roderick Docking, S. K. Chan, G. A. Taylor, D. L. Palmquist, S. D. Jackman, A. Nguyen, M. Li, H. Henderson, J. K. Janes, Y. Zhao, P. Pandoh, R. Moore, F. A. H. Sperling, D. P. W. Huber, I. Birol, S. J. M. Jones, and J. Bohlmann. 2013. Draft genome of the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, a major forest pest. Genome Biology 14:1-19.

Komada, S., Y. Kamae, M. Koyanagi, K. Tatewaki, E. Hassaneen, A. Saifullah, T. Yoshii, A. Terakita, and K. Tomioka. 2015. Green-sensitive opsin is the photoreceptor for photic entrainment of an insect circadian clock. Zoological Letters 1:1–10.

de Kort, C. A. D. 1990. Thirty-five years of diapause research with the Colorado potato beetle. Entomologia Experimentalis et Applicata 56:1–13.

Koštál, V. 2006. Eco-physiological phases of insect diapause. Journal of Insect Physiology 52:113–127.

Lampel, J., A. D. Briscoe, and L. T. Wasserthal. 2005. Expression of UV-, blue-, long-wavelength-sensitive opsins and melatonin in extraretinal photoreceptors of the optic lobes of hawkmoths. Cell and Tissue Research 321:443–458.

Lefevere, K. S., and C. A. D. de Kort. 1989. Adult diapause in the Colorado potato beetle, *Leptinotarsa decemlineata*: effects of external factors on maintenance, termination and post-diapause development. Physiological Entomology 14:299–308.

Liu, Z., and M. Friedrich. 2004. The Tribolium homologue of glass and the evolution of insect larval eyes. Developmental Biology 269:36–54.

Loeb, J. 1912. The Mechanistic Conception of Life. University of Chicago Press, Chicago, Illinois.

Lord, N. P., R. L. Plimpton, C. R. Sharkey, A. Suvorov, J. P. Lelito, B. M. Willardson, and S. M. Bybee. 2016. A cure for the blues: opsin duplication and subfunctionalization for short-wavelength sensitivity in jewel beetles (Coleoptera: Buprestidae). BMC Evolutionary Biology 16:1–17.

Martin, G. J., N. P. Lord, M. a. Branham, and S. M. Bybee. 2015. Review of the firefly visual system (Coleoptera: Lampyridae) and evolution of the opsin genes underlying color vision. Organisms Diversity & Evolution 15:513–526.

Numata, H., S. Shiga, and A. Morita. 1997. Photoperiodic Receptors in Arthropods. Zoological Science 14:187–197.

Pasquali, V., and V. Sbordoni. 2014. High variability in the expression of circadian rhythms in a cave beetle population. Biological Rhythm Research 45:925–939.

Porter, M. L. 2016. Beyond the eye: Molecular evolution of extraocular photoreception. Integrative and Comparative Biology 56:842–852.

Porter, M. L., J. R. Blasic, M. J. Bok, E. G. Cameron, T. Pringle, T. W. Cronin, and P. R. Robinson. 2012. Shedding new light on opsin evolution. Proceedings of the Royal Society B: Biological Sciences 279:3–14.

Provencio, I., G. Jiang, W. J. de Grip, W. P. Hayes, and M. D. Rollag. 1998. Melanopsin: An opsin in melanophores, brain, and eye. Proceedings of the National Academy of Sciences of the United States of America 95:340–345.

Provencio, I., I. R. Rodriguez, G. Jiang, W. P. Hayes, E. F. Moreira, and M. D. Rollag. 2000. A novel human opsin in the inner retina. The Journal of Neuroscience 20:600–605.

Ramirez, M. D., A. N. Pairett, M. S. Pankey, J. M. Serb, D. I. Speiser, A. J. Swafford, and T. H. Oakley. 2016. The last common ancestor of most bilaterian animals possessed at least nine opsins. Genome Biology and Evolution 8:3640–3652.

Richards, S. 2008. The genome of the model beetle and pest *Tribolium castaneum*. Nature 452:949–55.

Safranyik, L., and A. Carroll. 2006. The biology and epidemiology of the mountain pine beetle in lodgepole pine forests. Pages 3-66 (Chapter 1) in L. Safranyik and W.R. Wilson, editors. The mountain pine beetle: a synthesis of biology, management, and impacts on lodgepole pine. Natural Resources Canada, Canadian Forest Service, Pacific Forestry Centre, Victoria, British Columbia.

Safranyik, L., A. L. Carroll, J. Régnière, D. W. Langor, W. G. Riel, T. L. Shore, B. Peter, B. J. Cooke, V. G. Nealis, and S. W. Taylor. 2010. Potential for range expansion of mountain pine beetle into the boreal forest of North America. The Canadian Entomologist 142:415–442.

Safranyik, L., R. Silversides, L. McMullen, and D. Linton. 1989. An empirical approach to modeling the local dispersal of the mountain pine beetle (*Dendroctonus ponderosae* Hopk.) (Col., Scolytidae) in relation to sources of attraction, wind direction and speed. Journal of Applied Entomology 108:498–511.

Sandrelli, F., E. Tauber, M. Pegoraro, G. Mazzotta, P. Cisotto, J. Landskron, R. Stanewsky, A. Piccin, E. Rosato, M. Zordan, R. Costa, and C. P. Kyriacou. 2007. A molecular basis for natural selection at the timeless locus in *Drosophila melanogaster*. Science 316:1898–1900.

Saunders, D. S. 2012. Insect photoperiodism: Seeing the light. Physiological Entomology 37:207–218.

Saunders, D. S., and R. C. Bertossa. 2011. Deciphering time measurement: The role of circadian "clock" genes and formal experimentation in insect photoperiodism. Journal of Insect Physiology 57:557–566.

Schulz, W. D., U. Schlüter, and G. Seifert. 1984. Extraocular photoreceptors in the brain of *Epilachna varivestis* (Coleoptera, Coccinellidae). Cell and Tissue Research 236:317–320.

Sharkey, C. R., M. S. Fujimoto, N. P. Lord, S. Shin, D. D. McKenna, A. Suvorov, G. J. Martin, and S. M. Bybee. 2017. Overcoming the loss of blue sensitivity through opsin duplication in the largest animal group, beetles. Scientific Reports 7:1–10.

Shepherd, R. F. 1966. Factors influencing the orientation and rates of activity of *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae). The Canadian Entomologist 98:507–518.

Shimizu, I., Y. Yamakawa, Y. Shimazaki, and T. Iwasa. 2001. Molecular cloning of *Bombyx* cerebral opsin (Boceropsin) and cellular localization of its expression in the silkworm brain. Biochemical and Biophysical Research Communications 287:27–34.

Shintani, Y. 2011. Quantitative short-day photoperiodic response in larval development and its adaptive significance in an adult-overwintering cerambycid beetle, *Phytoecia rufiventris*. Journal of Insect Physiology 57:1053–1059.

Shintani, Y., Y. Ishikawa, and S. Tatsuki. 1996. Larval diapause in the yellow-spotted longicorn beetle, *Psacothea hilaris* (Pascoe) (Coleoptera: Cerambycidae). Applied Entomology and Zoology 31:489–494.

Spaethe, J., and A. D. Briscoe. 2005. Molecular characterization and expression of the UV opsin in bumblebees: three ommatidial subtypes in the retina and a new photoreceptor organ in the lamina. Journal of Experimental Biology 208:2347–2361.

Stecher, N., A. Stowasser, A. Stahl, and E. K. Buschbeck. 2016. Embryonic development of the larval eyes of the sunburst diving beetle, *Thermonectus marmoratus* (Insecta: Dytiscidae): A morphological study. Evolution and Development 18:216–228.

Stringer, I. A. N., and V. B. Meyer-Rochow. 2017. Flight activity of insects within a Jamaican cave: In search of the zeitgeber. American Microscopical Society 116:348–354.

Svaetichin, G., and E. F. MacNichol. 1958. Retinal mechanisms for chromatic and achromatic vision. Annals of the New York Academy of Sciences 74:385–404.

Tauber, E., M. Zordan, F. Sandrelli, M. Pegoraro, N. Osterwalder, C. Breda, A. Daga, A. Selmin, K. Monger, C. Benna, E. Rosato, C. P. Kyriacou, and R. Costa. 2007. Natural selection favors a newly derived timeless allele in *Drosophila melanogaster*. Science 316:1895–1898.

Tierney, S. M., S. J. B. Cooper, K. M. Saint, T. Bertozzi, J. Hyde, W. F. Humphreys, and A. D. Austin. 2015. Opsin transcripts of predatory diving beetles: a comparison of surface and subterranean photic niches. Royal Society Open Science 2:1–12.

Velarde, R. A., C. D. Sauer, K. K. O. Walden, S. E. Fahrbach, and H. M. Robertson. 2005. Pteropsin: A vertebrate-like non-visual opsin expressed in the honey bee brain. Insect Biochemistry and Molecular Biology 35:1367–1377.

Wald, G. 1968. Molecular basis of visual excitation. Science 162:230–239.

de Wilde, J., S .C. Duintjer, and L. Mook. 1959. Physiology of diapause in the adult Colorado beetle (*Leptinotarsa decemlineata* Say) – I. The photoperiod as a controlling factor. Journal of Insect Physiology 3:75–85.

Williams, K. S., and C. Simon. 1995. The Ecology, Behavior, and Evolution of Periodical Cicadas. Annual Review of Entomology 40:269–295.

Wolda, H. 1989. Seasonal Cues in Tropical Organisms. Rainfall? Not Necessarily! Oecologia 80:437–442.

Xiang, Y., Q. Yuan, N. Vogt, L. L. Looger, L. Y. Jan, and Y. N. Jan. 2010. Light-avoidance-mediating photoreceptors tile the *Drosophila* larval body wall. Nature 468:921–926.

Yuan, Q., D. Metterville, A. D. Briscoe, and S. M. Reppert. 2007. Insect cryptochromes: Gene duplication and loss define diverse ways to construct insect circadian clocks. Molecular Biology and Evolution 24:948–955.

CHAPTER 1 Figures

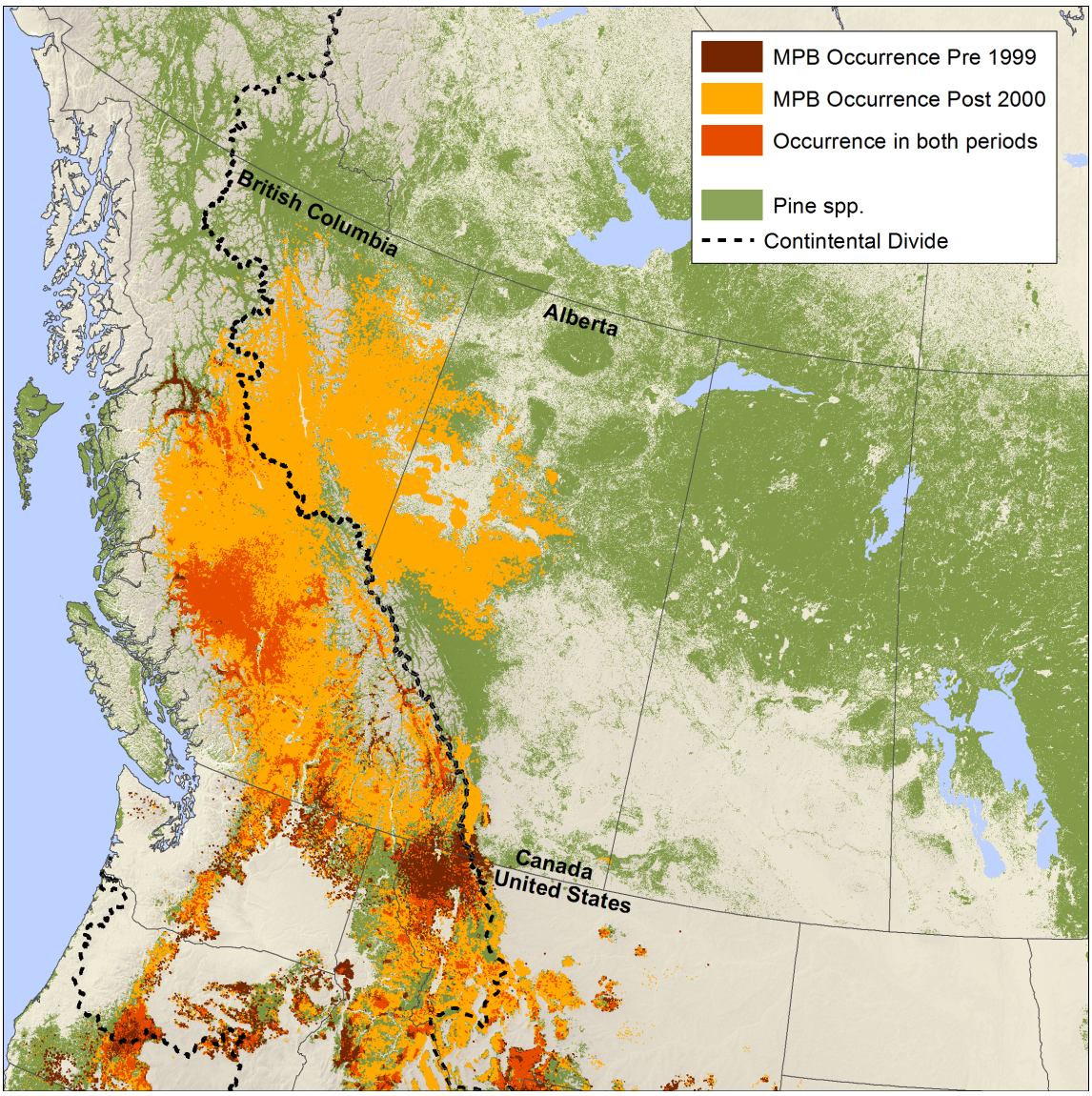


Figure 1. Occurrence map of mountain pine beetle in northwestern North America (adapted from Bleiker and Van Hezewijk 2016).

CHAPTER 2:

Behavioural and genetic studies of photosensitivity in larvae of the mountain pine beetle (*Dendroctonus ponderosae*)

INTRODUCTION

Vision and light perception in cryptozoic insects are characterized by eye reduction and loss, and retention of relatively few opsin genes (Jackowska et al. 2007, Friedrich et al. 2011, Wang et al. 2013, Futahashi et al. 2015, Tierney et al. 2015, Sharkey et al. 2017). As it is energetically expensive to produce and maintain unnecessary photoreceptor proteins and organs, the reduction of phototransduction machinery likely represents visual specialization or diminished reliance on vision in cryptic species (Fox et al. 2007, Tierney et al. 2015, Sharkey et al. 2017). Opsins, the photosensitive G protein-coupled receptor (GPCR) proteins that form the basis of all animal vision (Porter et al. 2012, Porter 2016), are reduced from the ancestral trichromatic condition in insects (three opsin orthologs) to 0-2 copies in cryptic species (Jackowska et al. 2007, Friedrich et al. 2011, Tierney et al. 2015). The mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Curculionidae, Scolytinae), with the exception of a short period of dispersal from natal trees to new hosts, completes its entire life cycle beneath the bark of trees (Safranyik and Carroll 2006). Photosensitivity in insects developing within subcortical tree tissues has been investigated, directly and indirectly, in very few systems to date (Shintani et al. 1996, Doležal and Sehnal 2007, Shintani 2011, Hustert and Mashaly 2013), as it is generally assumed that environmental light is unable to penetrate bark. Considering the extensive ecological and economic impacts of the mountain pine beetle in North America (Safranyik and Carroll 2006), the influence of light on immature *D. ponderosae* physiology deserves thorough investigation.

Long wavelength light and low-light adaptations in insects

Long wavelength opsins, which are sensitive to green light (480-600nm), may function in brightness detection and perception of day length in cryptozoic species (Jackowska et al. 2007, Friedrich et al. 2011, Tierney et al. 2015). Male *Ceratosolen solmsi* fig wasps with

vestigial eyes, for example, express a long-wavelength opsin in response to light, despite living their entire lives within fruits (Wang et al. 2013). Dragonfly larvae are benthic sedentary predators living in light-restricted aquatic environments, while the adults are highly visual active fliers, and these habitat differences are reflected in the opsins expressed at either life stage (Futahashi et al. 2015). Futahashi et al. (2015) found odonate larvae to express one blue and several long wavelength opsins (in contrast to the 11-30 visual opsin copies expressed in adults), and that in burrowing/pit-dwelling species, sole reliance on long wavelength light is reflected by the lack of blue opsin expression.

Beetles inhabit a wide variety of niches and have visual systems ranging from compound eyes and ocelli, to microphthalmy (eye reduction) and anophthalmy (complete loss of the eyes) (Martin et al. 2015). Caves are excellent systems for the study of photoreception in troglobiotic insects, due to the intricate relationships between photoreceptive organs and low-light gradients. The troglomorphic beetle *Ptomaphagus hirtus* (Cholevidae or Cholevinae within Leiodidae) has highly reduced eyes, while *Neaphaenops tellkampfii* (Carabidae) lacks both eyes and related brain regions completely (Friedrich et al. 2013). In species of diving beetle (Dytiscidae), convergent evolution of eyelessness occurred after repeated colonization of an aquifer system (Tierney et al. 2015). Eye reduction is also noted for adults of a cave-dwelling population of scolytid bark beetle, *Coccotrypes dactyliperda*, which inhabits palm fruit seed hosts that have been deposited by birds (Bright 1981).

The opsin repertoires of the cave beetle *Ptomaphagus hirtus* (Friedrich et al. 2011) and several species of diving beetle (Tierney et al. 2015) are exemplary of beetles adapted to low-light conditions. In *P. hirtus*, a troglomorphic carrion beetle that has long been considered blind despite presence of residual eyes, researchers found transcription of insect phototransduction and circadian clock genes, implying the presence of a reduced but intact visual system and the potential for reception of ambient light (Friedrich et al. 2011). The recovery of transcripts from only one long wavelength opsin indicates that *P. hirtus* likely lacks the ability to differentiate wavelengths and to form images. Opsins in

eyeless predatory diving beetles (Dytiscidae) from an Australian aquifer system range from relatively complex in surface species to absent or minimal in subterranean species (Tierney et al. 2015). Researchers recovered ultraviolet, long wavelength and C-type (non-visual in invertebrates) opsin transcripts from surface diving beetles, and a single long wavelength opsin copy from one of the three subterranean species, and report no opsin expression in the other two subterranean beetle species. While studies on cryptozoic Coleoptera have focused on adults, there have been very few investigations of photosensitivity in low-light adapted beetle larvae (Shintani et al. 1996, Doležal and Sehnal 2007, Hustert and Mashaly 2013).

Ultraviolet light and navigation in insects

While long wavelength light may be important in low-light environments, ultraviolet light (300-400nm) is used in insect navigation (Jackowska et al. 2007, Wang et al. 2013). Direct sunlight is high intensity and short wavelength skewed, with wavelengths as low as 291nm reaching the earth's surface (Edwards et al. 2008, Futahashi et al. 2015). Loss of ultraviolet sensitivity is virtually unknown in navigating insects with functional eyes (Wang et al. 2013). Desert ants (Formicidae) rely on ultraviolet light for visual navigation and individual foraging, having only coarse resolution in the compound eyes (Stone et al. 2014). Apparently the two spectral sensitivity peaks of the ants in the ultraviolet (350nm) and green (510nm) regions of the electromagnetic spectrum complement one another for highly effective horizon definition.

The effectiveness of compound eyes with coarse resolution, little to no colour vision, and both ultraviolet and long wavelength sensitive retinal receptors for navigation appears to extend to the scolytid beetles. Studies by Groberman and Borden (1981, 1982) on the retinal and behavioural spectral sensitivies of scolytid beetles (*Dendroctonus pseudotsugae, Ips paraconfusus* and *Trypodendron lineatum*) revealed two maxima for each species, in the blue and green regions of the visible spectrum. These data were collected, however, in the absence of ultraviolet light, and the noted "blue" sensitivity in scolytid beetles probably peaks in the ultraviolet (Sharkey et al. 2017). Groberman and Borden (1982) hypothesize shorter wavelengths (ultraviolet) are used for dispersal and

navigation, while longer wavelengths (green) are used for spatial processing and host selection in scolytids. In support of this, the Armand pine bark weevil (*Pissodes punctatus*, Subfamily Molytinae) uses ultraviolet sky polarization and sun position for navigation (Chen et al. 2012), while the red palm weevil (*Rhynchophorus ferrugineus,* Subfamily Dryophthorinae) navigates using ultraviolet-green contrast to define the sky from the horizon (Ilić et al. 2016).

Bark beetle phototaxis

In general, emerging scolytid beetles are reported to be positively phototactic to high intensity light, and only after a period of flight will the beetles become sensitive to other environmental stimuli and phototaxis reverse (Reid 1962, Shepherd 1966, Safranyik 1978, Groberman and Borden 1982, Safranyik et al. 1989). Visual cues are important for host tree location in weevils including the mountain pine beetle and the red palm weevil (*Rhynchophorus ferrugineus*), which are attracted to large, dark, high contrast objects (Shepherd 1966, Ilić et al. 2016). Newly emerged mountain pine beetles orient preferentially to spot sources of light, fixing on objects for flight orientation, and become negatively phototactic above 35°C and at the egg laying stage (Shepherd 1966, Safranyik 1978, Safranyik et al. 1989).

The only known documented case of phototaxis in subcortical tree-tissue developing weevil larvae was discovered accidentally while evaluating the rhythmic neuronal impulse patterns associated with crawling behaviour in the isolated central nervous system of the the red palm weevil (*R. ferrugineus*) (Hustert and Mashaly 2013). Researchers excised the ventral nerve chord from the anterior thoracic larval segments, spanning from the subesophageal ganglion to the last abdominal ganglion, and demonstrated its sensitivity to light. The authors also conducted behavioural assays and showed that live larvae exhibit negative phototaxis, turning laterally or crawling backwards in response to long wavelength light (450-660nm) exposure.

The mountain pine beetle

The mountain pine beetle (*Dendroctonus ponderosae* Hopkins) is a species of bark beetle belonging to the subfamily Scolytinae (Coleoptera: Curculionidae), which consists of numerous pests of forestry and agriculture found globally (Keeling et al. 2012). *D. ponderosae* is arguably the most significant biological threat to mature pine forests in western North America (Safranyik and Carroll, 2006; Safranyik et al. 2010). The current epidemic has extended east of the Rocky Mountains, historically an effective barrier to spread of the beetle, and is proceeding eastward across the boreal forest (Safranyik et al. 2010). Mountain pine beetle primarily utilizes lodgepole, ponderosa and western white pine as hosts (Safranyik and Carroll 2006), although it can colonize the majority of native pine species found within its range (Safranyik et al. 2010)..

Mountain pine beetle development is highly regulated by temperature, and occurs entirely within the subcortical tissues of host trees (Safranyik and Carroll 2006). *D. ponderosae* typically have a one year life cycle (univoltine), with adults emerging from hosts and attacking brood trees in July or August of each year. Larvae overwinter most successfully as late instars, resuming development and pupating into adults in June or July of the following year. Teneral adults usually mature under the bark for one to two weeks before emergence and dispersal. The under-bark habitat and development of the mountain pine beetle presents a unique system for investigating the role of light in bark beetle life histories.

Framework for study

The role of light in the biology of insect life stages developing within subcortical tree tissues is poorly investigated, presumably due to assumptions about the light microhabitats of cryptic organisms. We may gain insight into the ability of these insects to sense light based on the existence and activity of their photoreceptive organs and light transduction machinery. While mountain pine beetles acquire compound eyes during pupation, my SEM investigations of larvae have not revealed the presence of stemmata (Figure 1.), nor ocelli in adults (Figure 2.). This is consistent with the literature on

holometabolous insect larvae inhabiting low light habitats, as stemmata are known to be reduced in leaf mining caterpillars, and absent in flea larvae, wood-boring Symphyta larvae, and some species of bark beetle larvae (Gilbert 1994). Most adult beetles lack ocelli (Fleissner et al. 1993), and the absence of ocelli is noted for two other species of bark beetle, the red palm weevil (*Rhynchophorus ferrugineus*) (Ilić et al. 2016) and the Armand pine bark weevil (*Pissodes punctatus*) (Chen et al. 2012).

Because it is metabolically expensive to maintain unnecessary structures, the reduction and loss of exterior photoreceptor organs in certain insects may reflect reliance on general light perception, for which retinal reception may not be required, as opposed to vision and resolution (Fox et al. 2007, Tierney et al. 2015, Sharkey et al. 2017). The structuring of lens development by differentiation of photoreceptor cells is highly conserved in insects, however, and it is therefore likely that some element of vision is retained with eye reduction (Friedrich et al. 2011). Given the nearly universal effects of light on various insect life history traits (Jackowska et al. 2007, Saunders 2012), including adult mountain pine beetle dispersal (Shepherd 1966, Safranyik et al. 1989, it seems likely that bark beetle larvae would have retained some capacity for photoreception to fulfill functions that may be essential for survival. If photoreception in *D. ponderosae* larvae occurs, the insects must receive light information extraretinally, either directly in the brain or elsewhere in the body (Numata et al. 1997, Porter 2016).

Opsins are certainly expressed in adult beetle retinal receptors, and I suggest that long wavelength sensitive opsins in particular may also function in extraretinal photoreception in larvae. Ultraviolet light is considered damaging to plants, and thus external plant tissues generally have the ability to effectively screen ultraviolet wavelengths (Vogelmann 1993). Green (550nm) light penetrates farther into leaves than blue (450nm) and red (680nm) (Vogelmann 1993), suggesting that green wavelengths (referred to as "long wavelength" in the context of this thesis) may be representative of the under-bark light microhabitat.

The objectives of this study are to 1) determine, using a phototaxis assay, whether eyeless mountain pine beetle larvae exhibit behavioural responses to light and 2) expose the potential for photoreception in immature *D, ponderosae* at the molecular level, specifically by characterizing opsin expression across life stages and photoperiod treatments.

METHODS

Phototaxis experiment

I implemented a phototaxis experiment to determine whether mountain pine beetle larvae are able to receive and respond behaviourally to light. For the phototaxis assay, individual units called phloem sandwiches were constructed to provide larvae with a choice to move towards light or dark. Bark beetle larvae must bore through compressed phloem to move effectively, and thus fresh phloem sandwiched between two panes of glass approximately mimics the natural environment.

Larvae for the phototaxis experiment and subsequent control were sourced from four different lodgepole pine (*Pinus contorta var. latifolia)* trees, two felled on 24 September 2015 near Grande Prairie, Alberta (Tree 1 and 3 N54.55396° W118.71654°), and another two on 21 September 2016 outside Whitecourt, Alberta (HV2 N54.22197° W116.59433° and HB2 N54.28255° W116.59732°). Experimental larvae from the September 2015 bolts were held at -7.5°C for 10-13 months, conditions in which the insects would halt development and remain healthy until warming. These larvae were collected from under the bark, and placed on moist (distilled water) filter paper inside a glass petri dish sealed with parafilm, and left undisturbed for two to three days at room temperature to resume activity. Larvae for one final experiment run and all control assays were obtained from the September 2016 bolts, which were held at 0.3°C, once the majority of insects had reached their third or fourth instar. These insects were left undisturbed at room temperature for up to 24 hours before assaying.

To obtain fresh phloem for the experiment, we identified a suitable shore pine (*Pinus contorta* var. *contorta*) from Jordan River, Vancouver Island (N48.40119° W123.88644°), which was felled in late July 2016 and cut into bolts for transport to the Pacific Forestry Centre. The bolts were stored at 5.1°C or 6.0°C until needed to construct phloem sandwiches for experimentation. As needed for each run, the outer bark was shaved away from a green bolt and phloem squares (approximately 14 X 14cm) were defined by chiseling through to the sapwood.

To construct a sandwich (Figure 3.), a phloem square was peeled away from the sapwood and placed phloem side up in the centre of a large pane of glass (15 X 30cm) edged with black duct tape. Four 1.2 X 0.5cm oval-shaped punches were placed horizontally along the centre of the square, removing phloem from the bark in these areas. A third or fourth instar mountain pine beetle larva was placed into each punch. Phloem pieces were oriented such that the long axis of the punches was aligned with the grain of the tissue; this allowed larvae to mine against the grain in either direction, which is consistent with natural larval feeding galleries.

Two smaller square glass panes (15 X 15cm), one unaltered and the other coated with black duct tape, were selected to provide the light and dark treatments for each sandwich. These panels were placed on top of the phloem so that the edges butted against one another directly across the punches, effectively exposing half of each larva-containing punch to either light or dark. Each sandwich was held together by a combination of clear packing tape, black masking tape, two large elastic bands, and four 0.75" alligator clips. These sandwiches were designed to be level, and tight enough to encourage mining and minimize the wandering of larvae between the phloem and treatment pane (for more detailed assembly information, see Appendix Figure 1.). The panes used to construct phloem sandwiches were 0.6cm thick tempered glass (Oldcastle Glass, Langley BC), and did not filter ultraviolet light (Broco Glass Victoria BC retailer, personal communication 2016). The panels were cleaned with dish soap and rinsed with 70% ethanol before and between use to minimize fungal and bacterial contamination of the phloem and larvae. For each run, sandwiches were numbered 1 to 8 in order of construction.

I measured phloem temperatures beneath the light and dark panels in each sandwich across four experimental runs (32 units) (Figure 4.). Eight two channel temperature loggers [HOBO Pro v2 2X External Temperature Data Logger (Part # U23-003), Onset Computer Corporation, Bourne MA], set to record temperature every five minutes, were implemented consistently in corresponding phloem sandwich units and treatment panels. To incorporate temperature probes into a sandwich, a strip of bark and phloem was removed from each side of the phloem square and a logger probe inserted tightly into the

gap, so that the sensor end contacted the phloem directly, and the remainder of the metal probe was surrounded by phloem tissue (Figure 5.). Sandwich assembly was altered to accomodate the probes, including the placement of white duct tape on the underside of each panel, functioning to cover each probe lengthwise with a material of similar colour to phloem (adhesive backside of white masking tape) and prevent light from transferring directly to the metal probe.

Eight sandwiches were run at a time beneath a 35 X 57cm bank of two broad-spectrum fluorescent lights (SunBlaster 2' T5 HO 24W 6400K with NanoTech T5 Reflector Combo, SunBlaster Lighting #0900302) and within a white foam core frame, which functioned to contain and focus the light (Figures 6. and 7.). The 2' 24 Watt fluorescent bulbs are engineered to mimic natural sunlight as closely as possible, and emit small amounts of both UVB (280-320nm) and UVA (320-400nm) (Sang et al. 2015) radiation (SunBlaster Lighting 2017). The light panel was suspended at a height of 33.5cm above the sandwiches, resulting in a maximum light intensity (illuminance) of 4740 lux. I arranged the phloem sandwiches in four different grid configurations beneath the light panel; each configuration was run twice (eight experimental runs total) to evenly account for any geotaxis or directional preference of the larvae (Figure 8.).

Each group of eight sandwiches was run for 96 hours total. Upon completion, sandwiches were disassembled and larvae were scored for light or dark choice, defined as having mined or wandered away from the division in either direction (Figure 9.). Wandering larvae, including those that initiated mining outside the phloem punch, were counted as data points only if it was determined that phloem-glass contact was equal on both treatment sides. Active larvae that had not mined and made a choice at time of experiment completion (i.e. remained in the punch), that mined along the divider without making a light or dark choice, and larvae that died during the experiment were excluded from analysis. Galleries were measured to the nearest 0.25cm, and measurements recorded alongside larval choice.

I executed a control to assess the expected light to dark choice ratio of 1:1 in the absence of light within my experimental constructs. Sandwiches for four control runs were assembled using exactly the same methods as in the experiment, and two runs featured the addition of temperature probes. The sandwiches were kept covered with black plastic after construction and before experimentation to minimize confounding light stimuli. While the sandwiches were arranged beneath the light panel in four grid configurations as in the experiment, the lights were left off to produce control conditions for the larvae. The windowless treatment room appeared completely devoid of light once the door exterior had been sealed using large black plastic sheeting.

The accuracy and resolution of either channel from each temperature logger [HOBO Pro v2 2X External Temperature Data Logger (Part # U23-003), Onset Computer Corporation, Bourne MA] are reported by the manufacturer to be ± 0.21°C and ± 0.02°C, respectively. Although it is not reported, I was able to determine the precision of the logger channels implemented in each phloem sandwich using temperature data from the phototaxis control experiment. I calculated the standard deviations of the average temperatures, considering each channel measurement as an independent data point, for each of the two control runs separately. These values were averaged between the two runs (two channels per sandwich, 16 measurements per run) to reveal a mean standard deviation of ± 0.050°C among loggers. This value is one-quarter of the manufacturer reported accuracy, and when taken as a measurement of logger channel precision reveals that the equipment resolution is adequate for discerning temperature variation at the ascribed scale.

Data analysis

A chi-square analysis was used to determine whether the observed experimental larva choice frequencies differ significantly from the expected light to dark choice ratio under the null hypothesis (no effect of light treatment on larval choice). Expected frequencies were calculated using data from control larvae assayed in the absence of light. Mean gallery lengths for the light and dark treatments were analyzed using a t-test.

All temperature files were standardized to obtain average temperatures from midnight on the day of run initiation through to experiment completion (HOBOware Pro Version 3.7.4, Onset Computer Corporation, Bourne MA). I evaluated the effect of temperature variation between the light and dark panels from four experimental runs (32 sandwiches) using two different statistical analyses. First, I incorporated the temperature differences calculated for each sandwich into a binomial logistic model with corresponding choice data to test for an effect of temperature variation on larval choice. I also used a paired t-test to determine whether the mean recorded light and dark panel temperatures differed significantly from one another. These analyses were repeated for temperatures obtained from two control runs (16 sandwiches). All analyses were executed in RStudio (RStudio Team 2012, R Core Team 2015), and figures produced using ggplot2 (Wickham 2009).

Opsins – phylogenetic analysis

Identifying opsin sequences from the *D. ponderosae* genome

To exhaustively search the mountain pine beetle genome (Keeling et al. 2013) for opsin-like sequences, I used a bioinformatics server terminal (University of Victoria) to BLAST (tBLASTx) (Altschul et al. 1990) a complete and annotated opsin dataset for *Danio rerio* (41 sequences) against the *D. ponderosae* genome database. This survey yielded seventeen hits, which were further analyzed for opsin homology.

To approximately identify the coding regions of each identified sequence, I performed tBLASTx alignments using each opsin-like mountain pine beetle sequence as a nucleotide query, which was then automatically translated into all open reading frames and compared to the *Tribolium castaneum* nucleotide collection (NCBI Resource Coordinators 2017). The tBLASTx mountain pine beetle hit with the lowest E-value was assumed to be the coding region of a gene orthologous to a *T. castaneum* sequence encoding a specific GPCR protein. The isolated *D. ponderosae* coding regions were input as queries into BLASTp, and the most likely identity of each encoded protein was determined by evaluating annotated hits. Using this method, I was able to exclude all but two sequences as non-opsin GPCRs. The two definitive opsins were identified as a long-

wavelength opsin and an ultraviolet opsin, insect visual opsins belonging to the Opn4 subfamily (Provencio et al. 1998, 2000) that are annotated as hypothetical *D. ponderosae* proteins in the NCBI database.

Opsin phylogenetic tree (amino acid) construction

To situate the two *D. ponderosae* opsins within an evolutionary context, I constructed a phylogeny aligning the mountain pine beetle opsin amino acid sequences with *Tribolium castaneum, Apis mellifera, Drosophila melanogaster,* (select) *Danio rerio and Homo sapiens* Opn4 R-type (Provencio et al. 1998, 2000) and C-type opsins (summarized in Appendix Table 2). A MAFFT alignment (Katoh and Standley 2013) (Blosum62 cost matrix) was performed using Geneious version 7.1.9 (Kearse et al. 2012, http://www.geneious.com), and the phylogenetic substitution model selected using the Datamonkey webserver's (Delport et al. 2010, http://classic.datamonkey.org) Protein Model Selection and its default parameters. I constructed the phylogenetic tree using PhyML (Guindon et al. 2010) in SeaView version 4.6.2 (Gouy et al. 2010, http://doua.prabi.fr/software/seaview) with Blosum62+F (empirical) substitution, which was the top AIC output from Datamonkey Node support was determined using 1000 bootstrapped replicates. The phylogeny was formatted using FigTree version 1.4.3 (Rambaut 2016, http://tree.bio.ed.ac.uk/software/figtree/).

Opsins – gene expression analysis

The initial step in my investigation was to determine whether opsins are expressed in the pre-adult stages of *D. ponderosae* at all. Using qPCR and nucleotide sequencing, I discovered that the long-wavelength opsin is expressed in fourth instar larvae while the ultraviolet opsin is not. My samples were subsequently analyzed for the presence/ absence of long wavelength and ultraviolet opsin transcripts across life stages, and for levels of long wavelength opsin transcription in fourth instar larvae across photoperiods, and between body parts (head/body). Insects selected for RNA extraction and cDNA synthesis originated from either development experiment A or B (see CHAPTER 3), where beetles were reared from either eggs (B) or third/fourth instar larvae (A) to

emergence in growth chambers under consistent temperature (17.9°C to 18.9°C) and various photoperiods (light:dark) (A: 0:24, 10:14, 17:7, 24:0; B: 0:24, 17:7).

Three sets of experimental qPCR reactions were performed to evaluate opsin expression across life stages, treatments and body parts. Reaction sets included DNAse enzyme treated RNA controls corresponding to each cDNA sample to ensure the complete degradation of genomic DNA. Specific details about experimental samples used for each analysis are summarized in Appendix Table 1. RNA was extracted from selected samples using a phenol-chloroform extraction, and reverse transcribed to cDNA using standard reagents and protocol. Resultant cDNA from each respective sample was used for gene expression analysis.

I chose to normalize long wavelength opsin expression data by two different constitutively expressed reference genes, ribosomal protein subunit 3 (RPS3) and beta-actin (bAct) (Sang et al. 2015). These genes were identified in the mountain pine beetle genome, and coding regions defined, following the same procedure as for my initial list of opsin-like BLAST hits (see METHODS: Opsins – phylogenetic analysis).

Primer design

Primers were designed manually in Geneious version 9.1.5 (Kearse et al. 2012, http://www.geneious.com), aiming for a product size of 130 to 160 base pairs, a primer sequence length between 18 and 28 base pairs, and closely matched melt temperatures around 60°C (summarized in Table 1.). IDT OligoAnalyzer 3.1 (Integrated DNA Technologies 2016, https://www.idtdna.com/calc/analyzer) was used to evaluate potential primers and pairs for hairpin, self-dimer and heterodimer formation likelihood (-ΔG free energy), with a -ΔG of -9 used as a minimum threshold. Successful candidate primer pairs were then tested against the mountain pine beetle genome using Primer-BLAST to ensure that the primers are specific to one target. Primer sequences, melt temperatures and product sizes are summarized in Table 5.

Custom DNA oligos were ordered through IDT (Integrated DNA Technologies, Coralville, Iowa). Primers were dissolved to 200μMol in RNAse-free water, and further diluted into working stock solutions of 5μMol. Primer performance was evaluated based on the consistency of qPCR melt temperatures and curve peaks. Amplified sequence identify was confirmed for each primer pair through Sanger nucleotide sequencing (by Macrogen, Maryland or Sequetech, California).

RNA extraction and cDNA production

Samples were removed from -80°C and remained on ice during transport from the Pacific Forestry Centre to the University of Victoria. Specimens were processed individually using phenol-chloroform RNA extractions (TRIzol, Invitrogen, Thermo Fisher Scientific). Insects were ground separately in sterile RNAse/DNAse free 1.5mL microcentrifuge tubes with ~8-12 1mm zirconia/silica beads (in addition to some 0.5mm beads for smaller samples) using a mechanical bead-beater in 300-700μL Trizol (adjusted based on sample size) for 15 seconds, followed by addition of chloroform (amounting to 1/5th of the trizol volume). After subsequent incubation and centrifugation (15 minutes at 14000rpm), the RNA-containing aqueous layer was removed from each sample and placed into new tubes. This step was followed by a 100% isopropyl alcohol precipitation, and two 75% ethanol pellet washes. When deemed necessary due to small pellet size, 8μL of glycogen was added to each sample prior to precipitation to increase pellet visibility. At this stage, samples were frozen for between 24 and 48 hours at -20°C.

Pellets were then dried and dissolved/resuspended in 50μL or 100μL of RNAse free water. Extracted RNA was assessed for quality using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and associated software. 260/280 and 260/230 values were evaluated, and reported RNA concentrations used to produce dilutions of 1μg RNA in 8μL water (exception 0.25μg head and body RNA in 8μL water) in 0.2mL DNAse and RNAse free sterile PCR strip tubes (Axygen). Standardized RNA solutions were then subject to genomic DNA deactivation using DNAse 1 enzyme and buffer (incubated at 37°C for 30min), followed by heat deactivation using EDTA (incubated at

65°C for 10 minutes), following the manufacture's protocol (Thermofisher) using a MasterCycler Gradient Thermal Cycler (Eppendorf). 2μL of the RNA product was diluted and reserved at this point for negative DNAse-treated RNA controls.

Reverse transcription was performed using 5X All-In-One RT MasterMix [Applied Biological Materials Inc. (ABM)] in 20μL reactions, according to the manufacturers instructions (cycled at 25°C for 10min, 42°C for 15 min, 85°C for 5min, then moved immediately to ice). The cDNA products were each diluted in water by either 1:2, 1:3 or 1:5, and were stored at -20°C until qPCR analysis.

Standard curves

Standard curves for each of the three primer pairs to be used in relative quantification (LW, RPS3, bAct) were generated using cDNA pooled from four emerged adults. To assess primer efficiency across six orders of magnitude, curves were generated from four 10 fold dilutions of cDNA stocks. Each sample was run in triplicate alongside no template controls. Cq values were plotted in dilution series and both primer efficiencies and R^2 values were recorded.

qPCR

Quantitative real-time PCR (qPCR) analyses were performed at the University of Victoria (Perlman lab) using a CFX96 Touch Real-Time PCR Detection System (C1000 Touch Thermal Cycler, Bio-Rad). 96 well qPCR plates (0.2mL, Thermo Scientific Molecular) were designed to optimize comparisons among both reference and target genes, two or three replicates per sample per target, and two no template control replicated per primer pair. qPCR master mixes were prepared for each primer set using ABM EvaGreen 2X qPCR Master Mix (ABM), in quantities relative to the proportions required for 16μL reactions (including 2μL cDNA, according to manufacturer instructions). Plates were oriented and labeled, and a corresponding ABM EvaGreen thermocycle was engaged for amplification and quantitative fluorescence analysis (SYBR). Prior to cDNA synthesis, DNAse treated RNA was reserved from each

experimental sample and analyzed subsequently via qPCR (RPS3), alongside a positive control, to confirm the successful degradation of genomic DNA.

I analyzed cDNA from sixteen fourth instar larvae sampled from development experiment A, four larvae from each of the four photoperiods (0:24, 10:14, 17:7, 24:0 L:D h), for variation in long wavelength opsin expression relative to light treatment. Larvae were sampled after spending two weeks at treatment photoperiods, and were collected approximately mid-day during the photophase of the long and short day treatments. Three qPCR plates were consistently prepared, each featuring the same larval cDNA in duplicate and one of three primer pairs (LW, RPS3 or bAct). One cDNA sample (0:24 sample 2) was re-run in triplicate, for the LW target and RPS3, on a separate plate because the original RPS3 replicates were more than 0.5 cycles apart. Resulting long wavelength opsin data were normalized for analysis using either reference gene separately.

To identify the location of long wavelength opsin expression in the larval body, I analyzed cDNA from the heads and bodies of seven fourth instar larvae from development experiment A, three from 17:7 and four from 10:14 photoperiodic treatments. Heads were dissected away from the body of each larva, and processed separately in pairs into cDNA for analysis. One qPCR plate was prepared to analyze head and body pairing for long wavelength opsin and reference gene (RPS3 and bAct) amplification.

Long wavelength and ultraviolet opsin expression across life stages were analyzed via qPCR by presence/absence of transcription only, as relative quantification across samples from two separate experiments is not representative of an independent cohort. To evaluate long wavelength opsin expression across life stages, from egg to emerged adult, I analyzed cDNA from both development experiments A and B. cDNA from four individuals of each fourth instar larvae, pupae, teneral and emerged adults was selected from experiment A, and cDNA from two eggs, four of each first, second and third instar larvae from experiment B. Each life stage replicate was collected at the same sample

time, and all insects for expression profiling were reared under a long day (17:7 L:D h) photoperiod. Three qPCR plates featured cDNA from each sample replicate and one of three (LW, RPS3, bAct) primer pairs. One cDNA sample (TA-1) was re-run for both the target and reference genes due to initial cycle divergence (> 0.5 cycles apart).

The cDNA from experiment A that was assayed for long wavelength opsin expression was also used to investigate ultraviolet opsin expression across life stages (L4 to EA). One qPCR plate was run with cDNA in triplicate, while the presence/absence of ultraviolet opsin transcripts was determined by comparing technical replicate Cq values (at least two replicates P to EA < 0.5 cycles apart), and melt curve peaks with the known value for the previously sequenced UV product.

Data analysis

A maximum threshold of 0.5 cycle difference in Cq values for technical replicates was enforced for each sample. For samples run in triplicate, the two replicates with the closest Cq values were selected (lower values selected if equal). Relative expression of the long wavelength opsin was quantified and analyzed using the statistical methods outlined in the IDT PrimeTime qPCR Application Guide (Brookman-Amissah et al. 2015, page 58). Cq values and primer efficiencies, obtained from standard curves, were used to calculate R values (a ratio measurement of fold-change expression) of the target gene normalized by a reference gene. R values were calculated across plates, with the exception of head-body and replacement samples which were compared to the respective reference gene within the plate.

For comparison across photoperiods and body parts, R values were calculated relative to the Cq means for 17:7 and head samples, respectively. R values were calculated relative to emerged adult samples across life stages in experiment A, and third instar larvae in experiment B, separated to reflect the fact that the samples are derived from two separate experiments (egg to L3 from experiment B, L4 to EA from experiment A). Calculations and analyses for each data set were performed separately for the long wavelength opsin relative to either reference gene (RPS3 or bAct). Results yielding from corresponding

analyses were compared to one another for discrepancies in expression patterns, thus decreasing likelihood that any conclusions are based on the instability of a reference gene.

Data analyses were performed using RStudio (RStudio Team 2012, R Core Team 2015), and boxplots of logR values produced using ggplot2 (Wickham 2009), for comparison of long wavelength opsin expression levels across photoperiodic treatments and body parts. R values were log transformed prior to analysis, standard procedure in gene expression analysis due to the skew introduced into the data by calculation and standardization procedures. ANOVA analyses were performed to quantify variation in transcript levels in fourth instar larvae across photoperiods, for each of the two reference genes. A paired-t test was implemented to evaluate expression levels in the head versus body of fourth instar larvae, with samples from the two photoperiods pooled together.

RESULTS

Phototaxis experiment

Larvae selected dark microhabitats significantly more often than expected under the null hypothesis and therefore appeared to exhibit negative phototaxis (chi-squared test: $\chi^2_1 = 8.8327$: $p < 0.01$). Figure 10. shows the counts of 158 experimental insects by light preference (light n = 57, dark n = 101) across eight runs, two per grid configuration. Control larvae did not deviate from the 1:1 light to dark choice ratio within phloem sandwich units assayed in the absence of light, with 34 larvae choosing light and 37 choosing dark. The expected choice frequencies for experimental data were calculated using the control ratio of 34/37, and then compared to observed ratio of 57/101 using chi-square analysis.

Out of the 256 experimental larvae (8 runs of 32 insects), 158 (61.7%) exhibited distinct light preferences and produced data used for analysis (results outlined above), while 98 (38.3%) larvae did not generate data suitable for analysis. 54 (21.1%) of these larvae were active at the time of experiment completion, remaining in the phloem punch and not having mined in any direction, while 11 larvae (4.3%) had either mined exclusively along the divider (9 larvae) or mined their way back to the divider from elsewhere in the sandwich (2 larvae). 14 larvae (5.5%) died, and 19 (7.4%) went missing during the duration of the experiment. Some larvae that displayed preferences started mining in the opposite direction and then reversed, or mined along the divider for a distance before exhibiting a choice. An analysis of gallery lengths [light mean (SD) 2.91cm (1.65), dark 2.51cm (1.41)] does not provide support for an effect of treatment on larval mining distance (t-test: $t_{74} = -1.3816$: $p = 0.17$).

I found no effect of temperature variation between light and dark panels on larval choice in this experiment, using two separate tests to analyze results from 85 larvae across 32 sandwiches and four runs, one per grid configuration. The mean (SD) temperature of the dark panels was 23.807°C (1.055), while the mean for the light panels was 23.840°C (1.089), for an average difference 0.033°C (0.201) in favour of light. There was no

significant difference between light and dark panel temperature means when analyzed using a paired t-test (t_{31} = -0.92683: p = 0.36). Likewise, temperature difference between panels was non-significant when incorporated into a binomial logistic model with larval choice (p = 0.23 after removal of the non-significant "Run" term). When temperature model choice data were analyzed separately using chi-square analysis, the observed light to dark choice frequencies (light n = 30, dark n = 55) were significantly different from expected (chi-squared test: χ^2_1 = 5.4017: $p < 0.05$), reinforcing the fact that light-dark choice was significant where temperature was not. In combination, the output from these analyses indicate that temperature variation between light and dark treatments did not influence larval behaviour in my phototaxis assays.

"Light" and "dark" panel temperatures were obtained from two phototaxis control runs, amounting to 16 sandwiches. The mean (SD) temperature for the "dark" panels was 19.043°C (0.270), while the mean for the "light" panels was 19.010°C (0.275), revealing an average temperature difference of 0.033°C (0.055) in favour of "dark". This mean temperature difference was identical to the mean calculated from experimental temperatures, but in the opposite direction and with less variability. The minimal mean temperature difference was statistically significant (paired t-test: t_{15} = 2.4267: $p < 0.05$), but I do not consider it to be biologically relevant. I found no evidence to support an effect of temperature on the light/dark choice of 33 control larvae ("light" n = 15, "dark" n =18) when temperature differences were incorporated into a binomial model, after removal of the non-significant "Run" term (p = 0.25).

Opsins – phylogenetic analysis

The long wavelength and ultraviolet opsins identified in the mountain pine beetle genome were confirmed as insect visual opsins through the construction and analysis of an opsin phylogeny. The phylogeny pictured in Figure 11. [produced in SeaView version 4.6.2 (Gouy et al. 2010, http://doua.prabi.fr/software/seaview) and formatted in FigTree version 1.4.3 (Rambaut 2016, http://tree.bio.ed.ac.uk/software/figtree/)] features amino acid sequences of the two mountain pine beetle visual opsins, aligned with visual and non-visual insect (*Tribolium castaneum, Apis mellifera, Drosophila melanogaster*) and

vertebrate (*Danio rerio, Homo sapiens*) opsin sequences. The Opn4 R-type opsin clade containing the *D. ponderosae* sequences includes both insect visual opsins and vertebrate non-visual melanopsins (Provencio et al. 1998, 2000). The C-type opsin clade pictured includes the vertebrate visual opsins and insect non-visual opsins, including the *T. castaneum* non-visual c-opsin and the *A. mellifera* pteropsin. I have identified c-opsin orthologs in diving beetles and in several other insects families but the ortholog appears to have been lost in the mountain pine beetle.

I identified two hypothetical proteins encoded by the opsin-like regions of the whole-genome shotgun contigs (Keeling et al. 2013) in *D. ponderosae* as a long wavelength opsin and an ultraviolet opsin, as described above. The same hypothetical proteins were annotated as a predicted rhodopsin-like protein (XP_019757680.1), and a predicted ultraviolet-sensitive-like opsin protein (XP_019755582.1) through the NCBI eukaryotic genome annotation pipeline in January 2017 (NCBI Resource Coordinators 2017). Congruence between my identification of opsin-coding whole-genome shotgun contigs and the newly annotated sequences was confirmed and is summarized in Table 2. At the nucleotide level, the newly annotated rhodopsin-like/long wavelength opsin in *D. ponderosae* is 83% identical to *T. castaneum* rhodopsin 1/6-like, while the *D. ponderosae* ultraviolet-sensitive-like opsin is 71% similar to *T. castaneum* ultraviolet-sensitive isoform X1.

Opsins – gene expression analysis

Efficiencies for each of three primer sets (LW, RPS3, bAct), targeting transcripts from the long wavelength opsin gene and two reference genes, ribosomal protein subunit 3 and beta-actin respectively, were calculated from standard curves (Table 3.). Melt curve peaks for each amplified product, which varied within one degree (low resolution), are also summarized in Table 3. There was no DNA (RPS3) amplification in RNA controls (DNAse-treated) from any of my samples, indicating that all qPCR analysis of cDNA reflected levels of gene expression only.

Opsin expression across life stages

Long wavelength opsin transcripts were recovered from each sample and every mountain pine beetle life stage surveyed, including eggs (Table 4.). All technical replicates amplified before cycle 32, and the majority of products exhibited melt curve peaks of 87.5°C above the default threshold.

Increased long wavelength opsin expression in pupae and adults relative to larvae and eggs was predicted due to the development of compound eyes during metamorphosis. While mean Cq values for both reference genes were relatively constant across life stages [RPS3: L4 Cq mean (SD) 17.67 (0.249), pupae 17.88 (0.800), teneral adults 17.86 (0.407); bAct: L4 14.35 (0.292), pupae 15.64 (0.800), teneral adult 14.31 (0.729)], the long wavelength opsin mean Cq values for the same life stages decreased considerably from larva to adult [L4 28.84 (1.127), pupae 25.30 (0.576), teneral adult 22.74 (0.565)]. These data may be viewed as support for higher long wavelength opsin expression (lower average Cq values) in pupae and adults than in larvae, which is most logically interpreted as an increase in long wavelength opsin transcription associated with the development of compound eyes.

Ultraviolet opsin transcripts were recovered from pupae, teneral and emerged adults, but not (fourth instar) larvae (Table 4.). At least two of the three technical replicates for each P, TA and EA sample were < 0.5 cycles apart with consistent melt peaks, indicating expression of the ultraviolet opsin in these life stages. From evaluation of melt curve profiles, I determined that the ultraviolet opsin gene was not expressed in fourth instar larvae, although late amplification of non-specific products did occur in three out of four samples. Ultraviolet opsin expression was thus classified as absent in fourth instar larvae.

Long wavelength opsin expression in fourth instar larvae across photoperiods

Statistical analyses performed using either the RPS3 or bAct normalized dataset revealed a non-significant effect of photoperiod on long wavelength opsin expression (RPS3

ANOVA: $F_{3,12} = 0.826$: $p = 0.51$; bAct ANOVA: $F_{3,12} = 0.958$: $p = 0.44$). Figure 12. shows long wavelength opsin expression across photoperiods normalized using RPS3, while the data in Figure 13. were normalized using bAct.

Head versus body long wavelength opsin expression in fourth instar larvae

Results from analyses evaluating long wavelength opsin expression in the heads and bodies of fourth instar larvae revealed a significant difference in transcript levels between the two insect parts (RPS3 paired t-test: $t_6 = 2.709$: $p < 0.05$; bAct paired t-test: $t_6 = 2.9126$: $p < 0.05$) (Figures 14. and 15.). That the long wavelength opsin was expressed at detectable levels in the body as well as the head, however, was surprising given that the presence of photoreceptive proteins, cells or organs in the larval body was not anticipated.

DISCUSSION

Results from this study provide considerable support for photosensitivity in immature *D. ponderosae*, specifically through the identification of negative phototaxis and opsin gene expression in larvae. I found that mature larvae significantly preferred dark over light conditions when provided with a phototaxis choice assay. To explore the potential mechanisms for photosensitivity in larvae, I examined the insects for opsin expression. I identified two opsins in the mountain pine beetle genome, a long wavelength and an ultraviolet opsin. Long wavelength opsin transcripts were recovered from all life stages (egg, four larval instars, pupa, teneral and emerged adult), while ultraviolet opsin transcripts were recovered from pupae and adults but not from fourth instar larvae. I uncovered no difference in long wavelength opsin expression levels across photoperiods, but did detect higher expression in the larval head relative to the body.

Phototaxis

This research has documented negative phototaxis behaviour in larvae of the mountain pine beetle using a novel phloem sandwich assay. While positive phototaxis and reversal after a period of flight is known in adult *D. ponderosae* as well as other scolytid beetles (Reid 1962, Shepherd 1966, Safranyik 1978, Groberman and Borden 1982, Safranyik et al. 1989), negative phototaxis in weevil larvae has only been documented once in the literature (Hustert and Mashaly 2013). The accidental discovery by Hustert and Mashaly (2013) of negative phototaxis and green light sensitivity in the ventral nerve chord of red palm weevil (*Rhynchophorus ferrugineus*) larvae provides considerable support to my light avoidance data for *D. ponderosae* larvae. Both the red palm weevil and the mountain pine beetle, belonging to the same family but different subfamilies (Curculionidae: Dryophthorinae versus Scolytinae), inhabit subcortical tree tissues as larvae and likely encounter comparable light microhabitats.

Negative phototaxis has been reported for the eyeless invertebrates *Caenorhabditis elegans* (Edwards et al. 2008, Gong et al. 2016), larval *Drosophila* (Xiang et al. 2010, Kane et al. 2013) and the collembolan *Folsomia candida* (Fox et al. 2007). *C. elegans*

exhibits a strong negatively phototactic response to short wavelength light, particularly ultraviolet light, which is facilitated by the gustatory-related receptor protein LITE-1 (Edwards et al. 2008, Gong et al. 2016). Negative phototaxis in *Drosophila* larvae is essential to survival, and the insects must remain submerged in their food to avoid exposure to the elements and predators (Xiang et al. 2010). Rhodopsin expressing Bolwig organs and gustatory-related receptor protein (Gr28b) expressing class IV dendritic arborization neurons are each implicated in *Drosophila* larval phototaxis, the extent to which depends upon light intensity (Xiang et al. 2010, Kane et al. 2013, Porter 2016). The eyeless collembolan *Folsomia candida* is negatively phototactic to both white and ultraviolet light (Fox et al. 2007).

One can readily speculate about the fitness benefits of negative phototaxis in *D. ponderosae* and other species of weevil larvae maturing within tree tissues. While mountain pine beetle adults preferentially select trees with thick bark for reproduction, woodpeckers are responsible for considerable thinning of pine bark in the field (Amman and Cole 1983, reviewed in Safranyik and Carroll 2006). Woodpeckers prey on *D. ponderosae* larvae, pupae and adults and have indirect effects on the insects by removing and chipping bark, effectively exposing the beetles to desiccation, predation and parasitism. In addition to desiccation, exposure of larvae to direct sunlight could lead to lethal warming, as survival of immature mountain pine beetle decreases above 24°C. Ultraviolet radiation from the sun can be particularly damaging, as it is capable of causing fatal photo-oxidative reactions in animal cells (Edwards et al. 2008). A functional system for light detection throughout the larval body, and neuronal communication to generate a light avoidance response, would be extremely advantageous to mitigate the many potential negative effects of light exposure on *D. ponderosae* larvae living beneath the bark.

The cave beetle *Ptomaphagus hirtus* exhibits negative phototaxis that researchers speculate prevents the insects from exiting the cave and facing exposure to predators, while a lower light threshold could keep the beetles from moving further into the aphotic zone, which would lead to resource limitations (Friedrich et al. 2011). The authors

contend that long wavelength light perception may function to maintain a disphotic, nutrient rich habitat within the cave. While *D. ponderosae* larvae mine as deeply into the phloem as possible and are often found in crevices (personal observation), the phloem-sapwood interface functions as an effective barrier against deeper burrowing. Light may function in an intensity dependent manner to keep the larvae mining within thick and nutritious phloem.

The large disparity in brightness between the light and dark treatments in my phototaxis assays, in contrast to subtle observed variation in temperature, indicate that light is the most likely factor guiding larval behaviour in this experiment. Although I cannot exclude the possibility that other factors (including microscopic temperature variation or the effects of light on phloem chemistry, for example) may have influenced my data, I am confident that these results genuinely reflect negative phototaxis in eyeless larvae. It is also worth noting that due to a design constraint the dark panels in my phloem sandwich constructions were not entirely devoid of light, and therefore my results may not reveal the full extent of light avoidance by *D. ponderosae* larvae.

Opsins – phylogeny and gene expression

The recovery of two opsin transcripts, one copy of each ultraviolet and long wavelength sensitive orthologs, is consistent with my initial assessment of the *D. ponderosae* genome, subsequent NCBI annotations of proteins and nucleotide coding sequences, and with available literature (NCBI Resource Coordinators 2017, Sharkey et al. 2017). The lack of a blue sensitive opsin is described across Coleoptera (Sharkey et al. 2017), while due to insufficient functional characterization in beetles, the absence of a C-type opsin is subject to speculation.

Tribolium castaneum, a darkling beetle (Tenebrionidae) and stored grain pest, is a cryptozoic species that spends all post-embryonic stages inside the substrate, and similarly to *D. ponderosae* possesses single ultraviolet and long wavelength opsin paralogs (Jackowska et al. 2007). While *T. castaneum* encodes an ultraviolet opsin, orthologs are absent in another species of darkling beetle, *Tenebrio molitor*, two under-

bark developing insects, *Monochamus alternatus* (Cerambycidae) and *Dastarcus helophoroides* (Bothrideridae) (Sharkey et al. 2017), in addition to the troglobiotic carrion beetle *Ptomaphagus hirtus* (Friedrich et al. 2011) and several species of subterranean diving beetle Dytiscidae (Tierney et al. 2015). Based on genome sequence data (Richards et al. 2008), *T. castaneum* also possesses a C-type (ciliary) opsin that has not been functionally characterized (Liu and Friedrich 2008, NCBI Resource Coordinators 2017).

C-opsin orthologs are also found in the adult *Apis mellifera* brain (pteropsin) (Velarde et al. 2005) and two species of surface-dwelling diving beetles (Dytiscidae) (Tierney et al. 2015); however a copy is not apparent in the Asian longhorn beetle (*Anoplophora glabripennis*, Cerambycidae) genome (McKenna et al. 2016, tBLASTn). The function of a non-visual C-type opsin in beetles does not appear to have been experimentally investigated. The lack of a C-type opsin ortholog in both the *A. glabripennis* and *D. ponderosae* genomes could reflect the shared subcortical tree tissue habitats of the two species.

The unexpected discovery of long wavelength opsin transcripts in all mountain pine beetle life stages, including eggs and eyeless larvae, may reflect the importance of long wavelength opsin in low levels of light detection (Jackowska et al. 2007). In fact, Crandall and Hillis (1997) found no difference in the rate of rhosopsin sequence evolution between cave dwelling and surface species of crayfish, indicating that selective pressures maintaining long wavelength opsin function exist in the apparent absence of light. While long-wavelength opsins are increasingly being identified in animals living in low light conditions, including the cave beetle *Ptomaphagus hirtus* and several species of diving beetle (Friedrich et al. 2011, Tierney et al. 2015), this appears to be the first instance of opsin transcript recovery from bark beetle larvae. I contend that the long wavelength opsin may function in negative phototaxis, circadian clock entrainment, or developmental events in immature *D. ponderosae*.

In contrast to the long wavelength opsin, ultraviolet opsin transcripts were only recovered from mountain pine beetle pupae and adults and not from larvae (fourth instar), coincident with the development of compound eyes. Although I cannot exclude the possibility of undetected low levels of ultraviolet opsin transcription in eggs and larvae (third instar and younger not evaluated), the difference in expression is more likely to reflect variation in wavelength dependency throughout the life cycle. While both long wavelength (green) and ultraviolet light are likely to function in mountain pine beetle dispersal, ultraviolet wavelengths are more directly linked to navigation in weevils and other insects (Friedrich et al. 2011, Chen et al. 2012, Wang et al. 2013, Stone et al. 2014, Ilić et al. 2016). Although *D. ponderosae* likely exhibit only two sensitivity peaks (Groberman and Borden 1981, 1982) and lack trichromatic vision, the ultraviolet and long wavelength opsin expressing photoreceptors in the compound eyes should produce the contrast and coarse resolution necessary for dispersal and host selection (Stone et al. 2014). In comparison, the lack of stemmata and single long wavelength opsin expressed in *D. ponderosae* larvae suggests that the immature beetles do not require vision beneath the bark, but may rely upon the perception of long wavelength light.

Differential opsin expression in immature versus mature insect life stages, as identified in the mountain pine beetle, can also be observed in dragonflies and mosquitoes that share an aquatic larval stage (Futahashi et al. 2015, Jenkins and Muskavitch 2015). Dragonflies express a maximum of one blue and several long wavelength opsins in the larval stage, in comparison to the 11-30 visual opsin copies expressed in adults (Futahashi et al. 2015). In *Anopheles gambiae*, two different sets of long wavelength opsins are expressed in larvae and adults, while adults with compound eyes also exhibit increased blue and ultraviolet opsin expression (Jenkins and Muskavitch 2015). The emerald ash borer (*Agrilus planipennis*), a wood boring buprestid beetle, is thought to possess a larval specific long wavelength opsin copy (Lord et al. 2016). Evidence for life stage specific opsin transcription in both holometabolous (*Anopheles gambiae, Agrilus planipennis, D. ponderosae,*) and non-holometabolous (Odonata) insects may reflect the striking life history and habitat differences between the immature (aquatic, subcortical)

and mature stages (free flying, host colonizing) of these species (Futahashi et al. 2015, Jenkins and Muskavitch 2015, Lord et al. 2016).

I found no difference in long wavelength opsin expression across fourth instar larvae exposed to four different photoperiods (0:24, 10:14, 17:7, 24:0). This is not entirely unexpected, as the fluctuations in opsin expression levels that have been documented within specific insect life stages are diurnally cyclical and related to photoperiodic stimuli, not to photoperiod itself (Oba and Kainuma 2009, Wang et al. 2013, Komada et al. 2015). In the fig wasp *Ceratosolen solmsi*, the expression of most opsin genes varies rhythmically by time, light exposure and by sex (Wang et al. 2013). Researchers also found a relationship between time of day and long wavelength opsin expression in female fireflies (*Luciola cruciate*) (Oba and Kainuma 2009). In the cricket *Gryllus bimaculatus*, only the blue opsin exhibits daily expression rhythmicity (Komada et al. 2015).

Identifying the body tissues in which the long wavelength opsin is expressed in *D. ponderosae* could provide clues as to its function. I detected a significant difference in long wavelength opsin expression level by larval body part, indicating that transcripts are more abundant in the head but are also found in the body. Although contamination of the bodies with cerebral neurons cannot be excluded, the most logical interpretation of these data is that the opsin in expressed throughout the central nervous system, in both the brain and the nerve chord. Transcription of an insect visual opsin throughout the body in an eyeless bark beetle larva is suggestive of pleiotropic extraretinal functions. Immunohistology is a logical next step to identify the location(s) of long wavelength opsin expression in the larval body.

Both cerebral and visual opsins have been speculated to function in circadian entrainment and the reception of photoperiodic information in the brains of insects (Shimizu et al. 2001, Lampel et al. 2005, Spaethe and Briscoe 2005, Velarde et al. 2005, Tierney et al. 2015). While a circadian or photoperiodic role of the long wavelength opsin assumes that light is able to penetrate the bark and reach the developing larvae, expression in the optic lobes and other regions of the brain could provide support for a regulatory function.

Long wavelength (green) light sensitivity has been linked to photoperiodism in larvae of the silkworm *Bombyx mori*, which express an extraocular long wavelength sensitive opsin (boceropsin) in the brain (Shimizu et al. 2001). Adult hawkmoths (Sphingidae) express a long wavelength opsin in the optic lobes that is speculated to function in circadian entrainment (Lampel et al. 2005). In the cricket *Gryllus bimaculatus*, a long wavelength opsin expressed in the compound eyes is directly responsible for circadian clock entrainment (Komada et al. 2015).

If long wavelength opsin expression is situated within the *D. ponderosae* brain, in which region(s) may transcripts be localized? While the ancestral condition in beetle larvae is six pairs of stemmata (Gilbert 1989), the mountain pine beetle are lacking stemmata completely. It is possible that the stemmata organs were internalized during bark beetle evolution in a manner similar to the retention of larval stemmata during metamorphosis in holometabolous insects (Gilbert 1994, Felisberti et al. 1997). If this is the case, the photoreceptive organ or cells are likely to be found in the optic lobes of *D. ponderosae* larvae (Fleissner et al. 1993, Gilbert 1994, Felisberti et al. 1997, Spaethe and Briscoe, 2005). Internalized adult stemmata in beetles are green light-sensitive (Felisberti et al. 1997), which corresponds to the presumed spectral sensitivity of the *D. ponderosae* long wavelength opsin. Mountain pine beetle larvae could also possess stemmata-independent extraocular photoreceptor organs in the optic lobes, as described for adult *Pachymorpha sexguttata* (Fleissner et al. 1993). Regardless of the site of extraocular photoreception in the *D. ponderosae* larval brain, a possible circadian regulatory function cannot be disregarded (Fleissner et al. 1993, Lampel et al. 2005).

Long wavelength opsin expression in the *D. ponderosae* larval body could indicate a functional role in the light avoidance behaviour observed in this study. In larvae of the red palm weevil, *Rhynchophorus ferrugineus* (Curculionidae, Dryophthorinae), researchers found that the isolated ventral nerve chord (from subesophageal ganglion to the last abdominal ganglion and excluding the brain) is sensitive to light despite the lack of obvious photoreceptors (Hustert and Mashaly 2013). Exposure of the nerve chord to green (blue-yellow) light accelerates the reported impulse patterns from 0.05 to 0.5Hz,

leading the authors to conclude that photoreceptive cells or an organ must be present in the nerve chord itself. As negative phototaxis was also observed in live *R. ferrugineus* larvae, a similar system may exist in *D. ponderosae* to facilitate negative phototaxis via long wavelength opsin photoreception in larvae.

Some precedent for intraganglionic photoreception in the central nervous systems of invertebrates can be found in the literature, for weevil larvae (described above), as well as for crayfish and the butterfly *Papilio xuthus* (Arikawa et al. 1991, Hustert and Mashaly 2013). The photoreceptive interneuron found in crayfish, which is connected to the caudal photoreceptor, functions in negative phototaxis and circadian regulation (summarized in Arikawa et al. 1991). In *P. xuthus*, photoreceptive neurons located on the genitalia of adult butterflies connect directly to an extraocular photoreceptor situated in the last abdominal ganglion of the central nervous system (Arikawa et al. 1991). These examples of central nervous system light-sensitivity in invertebrates, particularly the activation of *R. ferrugineus* ventral nerve chord by green light, combine with my expression data to form a convincing case for a functional role of CNS-expressed long wavelength opsins in *D. ponderosae* larval phototaxis (Arikawa et al. 1991, Hustert and Mashaly 2013). It is also possible that unidentified gustatory-related receptor proteins in mountain pine beetle larvae could function in negative phototaxis (Xiang et al. 2010, Gong et al. 2016, Porter 2016).

The identification of long wavelength opsin expression in mountain pine beetle eggs could potentially indicate a critical function in development or circadian regulation. The two egg samples extracted for RNA were not classified by stage [as defined by Reid and Gates (1970)] prior to processing, and we cannot exclude the possibility that hatching of first instar larvae with presumed photoreceptive abilities was imminent. In the diving beetle *Thermonectus marmoratus*, however, eye primordia become apparent in the embryo at 10% of development (Stecher et al. 2016), suggesting that long wavelength opsin expression in immature *D. ponderosae* egg stages is possible. A long wavelength opsin ortholog was identified in the only insect egg transcriptome available in the NCBI database (NCBI Resource Coordinators 2017), for the moth *Athetis lepigone* (Institute of

Millet Crops, Hebei Academy of Agriculture and Forestry Sciences 2013), providing an interesting parallel to my data.

Opsins expressed in zebrafish eggs have critical developmental functions (Friedmann et al. 2015, Hang et al. 2016). The vertebrate ancient opsin is expressed extraretinally early in zebrafish embryogenesis, coinciding with central nervous system activity that is essential to development (Friedmann et al. 2015). Green light (504nm) stimulation of the opsin-expressing spinal neurons results in maximum suppression of this behaviour. Light apparently provides the first sensory input to the spinal chord, as embryos at this stage have not developed retinal photoreceptors or responses to other stimuli. Adult zebrafish females with vertebrate ancient opsin knockouts produce eggs with abnormal chorion formation that die within 26 hours of fertilization (Hang et al. 2016). If a critical development role of the long wavelength opsin in *D. ponderosae* eggs exists, it does not appear to require light input to function, as eggs reared to emerged adults in the dark develop normally (see CHAPTER 3).

Future directions

This project has exposed considerable potential for the exploration of bark beetle-light interactions and the ecological implications of these relationships. Future work should focus on elucidating the function of the long wavelength opsin in larvae and eggs, as well as locating the site(s) of opsin expression in larval body tissues. RNA interference is a highly effective technique used to evaluate gene function in *T. castaneum*, producing a systemic effect that spreads effectively throughout all tissues across life stages (Posnien et al. 2009). When administered to female pupae or adults, the gene knockout effect is transmitted to the next generation. One could harness this technology to produce mountain pine beetle adults with long wavelength opsin gene knockouts, and evaluate survival of their offspring. This experiment could clarify whether the long wavelength opsin serves a critical developmental function in *D. ponderosae* eggs.

To determine whether the long wavelength opsin is responsible for negative phototaxis in *D. ponderosae* larvae, two experiments come to mind. My phototaxis assays could be replicated with cinematic filter screening of the broad-spectrum lights, allowing only

certain wavelengths to reach the experimental larvae. If larvae are biased in their avoidance of ultraviolet or long wavelength light, we may conclude that either the long wavelength opsin or an unidentified ultraviolet light photoreceptor (possibly a gustatory-related receptor protein) is likely governing the phototactic response. Alternatively, RNAi could be implemented to knockout long wavelength opsin expression in mountain pine beetle larvae, which would be subsequently assayed for phototaxis. Either of these experiments could reveal the specific wavelengths responsible for stimulating light avoidance in larvae of the mountain pine beetle.

In sum, this study has generated considerable support for the existence of functional photoreception in *D. ponderosae* larvae living beneath the bark of trees. The long wavelength opsin expressed in larvae could have pleiotropic functions including mediation of phototaxis, reception of photoperiodic information, or play a fundamental role in development. The detection of negative phototaxis and long wavelength opsin expression in eyeless mountain pine beetle larvae suggest that light perception is functional in the cryptozoic under-bark habitat and may contribute to the survival of immature beetles.

REFERENCES

Altschul S.F., W. Gish, W. Miller, E. W. Myers, D. J. Lipman. 1990. Basic local alignment search tool. Journal of Molecular Biology 215:403–410.

Amman, G. D. and W. E. Cole. 1983. Mountain pine beetle dynamics in lodgepole pine forests. Part II: Population dynamics. USDA Forest Service, Intermountain Forest and Range Experiment Station, General Technical Report INT-145: 1-59.

Arikawa, K., K. Uchiumi, and E. Eguchi. 1991. Extraocular photoreceptors in the last abdominal ganglion of a swallowtail butterfly, *Papilio xuthus*. Naturwissenschaften 78:82–84.

Bright, D. E. 1981. Eye reduction in a cavernicolous population of *Coccotrypes dactyliperda* Fabricius (Coleoptera: Scolytidae). The Coleopterists Bulletin 35:117–120.

Brookman-Amissah, N., H. Packer, E. Prediger, J. Sabel, S. Gunstream, J. Hellemans, A. Menezes, B. Owens, S. Rose, R. Sander, and J. Vandesompele. 2015. PrimeTime qPCR Application Guide: Experimental Overview, Protocol, Troubleshooting. Fourth Edition. Integrated DNA Technologies (IDT), Coralville, Iowa.

Chen, Y., C. W. Luo, R. P. Kuang, H. W. Li, Z. Chen, and Y. J. Liu. 2012. Phototactic behavior of the Armand pine bark weevil, *Pissodes punctatus*. Journal of Insect Science 13:1–10.

Crandall, K. A., and D. M. Hillis. 1997. Rhodopsin evolution in the dark. Nature 387:667–668.

Delport, W., Poon, A. F., Frost, S. D. W and Kosakovsky Pond, S. L. 2010. Datamonkey 2010: a suite of phylogenetic analysis tools for evolutionary biology. Bioinformatics 26(19): 2455–2457.

Doležal, P., and F. Sehnal. 2007. Effects of photoperiod and temperature on the development and diapause of the bark beetle *Ips typographus*. Journal of Applied Entomology 131:165–173.

Edwards, S. L., N. K. Charlie, M. C. Milfort, B. S. Brown, C. N. Gravlin, J. E. Knecht, and K. G. Miller. 2008. A novel molecular solution for ultraviolet light detection in *Caenorhabditis elegans*. PLoS Biology 6:1715–1729.

Felisberti, F., D. F. Ventura, and H. Hertel. 1997. Cerebral extraocular photoreceptors in beetles. Comparative Biochemistry and Physiology 118A:1353–1357.

Fleissner, G., G. Fleissner, and B. Frisch. 1993. A new type of putative non-visual photoreceptors in the optic lobe of beetles. Cell and Tissue Research 273:435–445.

Fox, G. L., C. a Coyle-Thompson, P. F. Bellinger, and R. W. Cohen. 2007. Phototactic responses to ultraviolet and white light in various species of Collembola, including the eyeless species, *Folsomia candida*. Journal of Insect Science 7:1–12.

Friedmann, D., A. Hoagland, S. Berlin, and E. Y. Isacoff. 2015. A spinal opsin controls early neural activity and drives a behavioral light response. Current Biology 25: 69–74.

Friedrich, M., R. Chen, E. K. Buschbeck, S. Peck, M. Friedrich, R. Chen, E. K. Buschbeck, and S. Peck. 2013. A functional visual system in the cave beetle *Ptomaphagus hirtus*. Mammoth Cave National Park's 10th Research Symposium: Celebrating the Diversity of Research in the Mammoth Cave Region:41–43.

Friedrich, M., R. Chen, B. Daines, R. Bao, J. Caravas, P. K. Rai, M. Zagmajster, and S. B. Peck. 2011. Phototransduction and clock gene expression in the troglobiont beetle *Ptomaphagus hirtus* of Mammoth cave. Journal of Experimental Biology 214:3532–3541.

Futahashi, R., R. Kawahara-Miki, M. Kinoshita, K. Yoshitake, S. Yajima, K. Arikawa, and T. Fukatsu. 2015. Extraordinary diversity of visual opsin genes in dragonflies. Proceedings of the National Academy of Sciences of the United States of America 112:E1247–E1256.

Gilbert, C. 1989. Visual determinants of escape in tiger beetle larvae (Cicindelidae). Journal of Insect Behavior 2:557–574.

Gilbert, C. 1994. Form and function of stemmata in larvae of holometabolous insects. Annual Review of Entomology 39:323–349.

Gong, J., Y. Yuan, A. Ward, L. Kang, B. Zhang, Z. Wu, J. Peng, Z. Feng, J. Liu, and X. Z. S. Xu. 2016. The *C. elegans* taste receptor homolog LITE-1 is a photoreceptor. Cell 167:1252–1263.

Gouy, M. Guindon, S. and Gascuel., O. 2010. SeaView version 4 : a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Molecular Biology and Evolution 27(2):221-224.

Groberman, L. J., and J. H. Borden. 1981. Behavioral response of *Dendroctonus pseudotsugae* and *Trypodendron lineatum* (Coleoptera: Scolytidae) to selected wavelength regions of the visible spectrum. Canadian Journal of Zoology 59:2159–2165.

Groberman, L. J., and J. H. Borden. 1982. Electrophysiological response of *Dendroctonus pseudotsugae* and *Ips paraconfusus* (Coleoptera: Scolytidae) to selected wavelength regions of the visible spectrum. Canadian Journal of Zoology 60:2180–2189.

Guindon, S., J.-F. Dufayard, V. Lefort, M. Anisimova, W. Hordijk, and O. Gascuel. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: asessing the performance of PhyML 2.0. Systematic Biology 59:307–321.

Hang, C. Y., S. Moriya, S. Ogawa, and I. S. Parhar. 2016. Deep brain photoreceptor (val-opsin) gene knockout using CRISPR/Cas affects chorion formation and embryonic hatching in the zebrafish. PLoS ONE 11:1–20.

Hustert, R., and A. M. Mashaly. 2013. Spontaneous behavioral rhythms in the isolated CNS of insects - Presenting new model systems. Journal of Physiology (Paris) 107:147–151.

Ilić, M., P. Pirih, and G. Belušič. 2016. Four photoreceptor classes in the open rhabdom eye of the red palm weevil, *Rynchophorus ferrugineus* Olivier. Journal of Comparative Physiology A 202:203–213.

Jackowska, M., R. Bao, Z. Liu, E. C. McDonald, T. A. Cook, and M. Friedrich. 2007. Genomic and gene regulatory signatures of cryptozoic adaptation: Loss of blue sensitive photoreceptors through expansion of long wavelength-opsin expression in the red flour beetle *Tribolium castaneum*. Frontiers in Zoology 4:1–11.

Jenkins, A. M., and M. A. T. Muskavitch. 2015. Crepuscular behavioral variation and profiling of opsin genes in *Anopheles gambiae* and *Anopheles stephensi* (Diptera: Culicidae). Journal of Medical Entomology 52:296–307.

Kane, E. a, M. Gershow, B. Afonso, I. Larderet, M. Klein, A. R. Carter, B. L. de Bivort, S. G. Sprecher, and A. D. T. Samuel. 2013. Sensorimotor structure of *Drosophila* larva phototaxis. Proceedings of the National Academy of Sciences of the United States of America 110:E3868–E3877.

Katoh, K., and D. M. Standley. 2013. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Molecular Biology and Evolution 30:772–780.

Kearse, M., R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, S. Buxton, A. Cooper, S. Markowitz, C. Duran, T. Thierer, B. Ashton, P. Mentjies, and A. Drummond. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28(12): 1647-1649.

Keeling, C. I., H. Henderson, M. Li, M. Yuen, E. L. Clark, J. D. Fraser, D. P. W. Huber, N. Y. Liao, T. Roderick Docking, I. Birol, S. K. Chan, G. A. Taylor, D. Palmquist, S. J. M. Jones, and J. Bohlmann. 2012. Transcriptome and full-length cDNA resources for the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, a major insect pest of pine forests. Insect Biochemistry and Molecular Biology 42:525–536.

Keeling, C. I., M. M. S. Yuen, N. Y. Liao, T. Roderick Docking, S. K. Chan, G. A. Taylor, D. L. Palmquist, S. D. Jackman, A. Nguyen, M. Li, H. Henderson, J. K. Janes, Y. Zhao, P. Pandoh, R. Moore, F. A. H. Sperling, D. P. W. Huber, I. Birol, S. J. M. Jones, and J. Bohlmann. 2013. Draft genome of the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, a major forest pest. Genome Biology 14:1-19.

Komada, S., Y. Kamae, M. Koyanagi, K. Tatewaki, E. Hassaneen, A. Saifullah, T. Yoshii, A. Terakita, and K. Tomioka. 2015. Green-sensitive opsin is the photoreceptor for photic entrainment of an insect circadian clock. Zoological Letters 1:1–10.

Lampel, J., A. D. Briscoe, and L. T. Wasserthal. 2005. Expression of UV-, blue-, long-wavelength-sensitive opsins and melatonin in extraretinal photoreceptors of the optic lobes of hawkmoths. Cell and Tissue Research 321:443–458.

Liu, Z. and Friedrich, M. 2008. The ciliary type photoreceptor cells in the primitive insect flour beetle *Tribolium castaneum*. Unpublished, direct submission to GenBank. Wayne State University, Detroit, MI.

Lord, N. P., R. L. Plimpton, C. R. Sharkey, A. Suvorov, J. P. Lelito, B. M. Willardson, and S. M. Bybee. 2016. A cure for the blues: opsin duplication and subfunctionalization for short-wavelength sensitivity in jewel beetles (Coleoptera: Buprestidae). BMC Evolutionary Biology 16:1–17.

Martin, G. J., N. P. Lord, M. a. Branham, and S. M. Bybee. 2015. Review of the firefly visual system (Coleoptera: Lampyridae) and evolution of the opsin genes underlying color vision. Organisms Diversity & Evolution 15:513–526.

McKenna et al. 2016. Genome of the Asian longhorned beetle (*Anoplophora glabripennis*), a globally significant invasive species, reveals key functional and evolutionary innovations at the beetle-plant interface. Genome Biology 17:227.

NCBI Resource Coordinators. 2017. Database resources of the National Center for Biotechnology Information. Nucleic Acids Research.: 45, D12-D17.

Numata, H., S. Shiga, and A. Morita. 1997. Photoperiodic Receptors in Arthropods. Zoological Science 14:187–197.

Oba, Y., and T. Kainuma. 2009. Diel changes in the expression of long wavelength-sensitive and ultraviolet-sensitive opsin genes in the Japanese firefly, *Luciola cruciata*. Gene 436:66–70.

Porter, M. L. 2016. Beyond the eye: Molecular evolution of extraocular photoreception. Integrative and Comparative Biology 56:842–852.

Porter, M. L., J. R. Blasic, M. J. Bok, E. G. Cameron, T. Pringle, T. W. Cronin, and P. R. Robinson. 2012. Shedding new light on opsin evolution. Proceedings of the Royal Society B: Biological Sciences 279:3–14.

Posnien, N., J. Schinko, D. Grossmann, T. D. Shippy, B. Konopova, and G. Bucher. 2009. RNAi in the red flour beetle (*Tribolium*). Cold Spring Harbor Protocols 4:1–7.

Provencio, I., G. Jiang, W. J. de Grip, W. P. Hayes, and M. D. Rollag. 1998. Melanopsin: An opsin in melanophores, brain, and eye. Proceedings of the National Academy of Sciences of the United States of America 95:340–345.

Provencio, I., I. R. Rodriguez, G. Jiang, W. P. Hayes, E. F. Moreira, and M. D. Rollag. 2000. A novel human opsin in the inner retina. The Journal of Neuroscience 20:600–605.

R Core Team. 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Reid, R. W., and H. Gates. 1970. Effect of temperature and resin on hatch of eggs of the mountain pine beetle (*Dendroctonus ponderosae*). The Canadian Entomologist 102:617–622.

Richards et al., *Tribolium* Genome Sequencing Consortium. 2008. The genome of the model beetle and pest *Tribolium castaneum*. Nature 452:949–55.

RStudio Team. 2012. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA. URL http://www.rstudio.com/.

Safranyik, L. 1978. Effects of climate and weather on mountain pine beetle populations. Pages 77–84. Theory and practice of mountain pine beetle mamagement in lodgepole pine forests. Moscow, Idaho.

Safranyik, L., and A. Carroll. 2006. The biology and epidemiology of the mountain pine beetle in lodgepole pine forests. Pages 3-66 (Chapter 1) in L. Safranyik and W.R. Wilson, editors. The mountain pine beetle: a synthesis of biology, management, and impacts on lodgepole pine. Natural Resources Canada, Canadian Forest Service, Pacific Forestry Centre, Victoria, British Columbia.

Safranyik, L., A. L. Carroll, J. Régnière, D. W. Langor, W. G. Riel, T. L. Shore, B. Peter, B. J. Cooke, V. G. Nealis, and S. W. Taylor. 2010. Potential for range expansion of mountain pine beetle into the boreal forest of North America. The Canadian Entomologist 142:415–442.

Safranyik, L., R. Silversides, L. McMullen, and D. Linton. 1989. An empirical approach to modeling the local dispersal of the mountain pine beetle (*Dendroctonus ponderosae* Hopk.) (Col., Scolytidae) in relation to sources of attraction, wind direction and speed. Journal of Applied Entomology 108:498–511.

Sang, W., L. He, X.-P. Wang, K. Zhu-Salzman, and C.-L. Lei. 2015. Evaluation of reference genes for RT-qPCR in *Tribolium castaneum* (Coleoptera: Tenebrionidae) under UVB stress. Environmental Entomology 44:418–425.

Saunders, D. S. 2012. Insect photoperiodism: Seeing the light. Physiological Entomology 37:207–218.

Sharkey, C. R., M. S. Fujimoto, N. P. Lord, S. Shin, D. D. McKenna, A. Suvorov, G. J. Martin, and S. M. Bybee. 2017. Overcoming the loss of blue sensitivity through opsin duplication in the largest animal group, beetles. Scientific Reports 7:1–10.

Shepherd, R. F. 1966. Factors influencing the orientation and rates of activity of *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae). The Canadian Entomologist 98:507–518.

Shimizu, I., Y. Yamakawa, Y. Shimazaki, and T. Iwasa. 2001. Molecular cloning of *Bombyx* cerebral opsin (Boceropsin) and cellular localization of its expression in the silkworm brain. Biochemical and Biophysical Research Communications 287: 27–34.

Shintani, Y. 2011. Quantitative short-day photoperiodic response in larval development and its adaptive significance in an adult-overwintering cerambycid beetle, *Phytoecia rufiventris*. Journal of Insect Physiology 57:1053–1059.

Shintani, Y., Y. Ishikawa, and S. Tatsuki. 1996. Larval diapause in the yellow-spotted longicorn beetle, *Psacothea hilaris* (Pascoe) (Coleoptera: Cerambycidae). Applied Entomology and Zoology 31:489–494.

Spaethe, J., and A. D. Briscoe. 2005. Molecular characterization and expression of the UV opsin in bumblebees: three ommatidial subtypes in the retina and a new photoreceptor organ in the lamina. Journal of Experimental Biology 208:2347–2361.

Stecher, N., A. Stowasser, A. Stahl, and E. K. Buschbeck. 2016. Embryonic development of the larval eyes of the sunburst diving beetle, *Thermonectus marmoratus* (Insecta: Dytiscidae): A morphological study. Evolution and Development 18: 216–228.

Stone, T., M. Mangan, P. Ardin, and B. Webb. 2014. Sky segmentation with ultraviolet images can be used for navigation. Proceedings Robotics: Science and Systems: 1–10.

Tierney, S. M., S. J. B. Cooper, K. M. Saint, T. Bertozzi, J. Hyde, W. F. Humphreys, and A. D. Austin. 2015. Opsin transcripts of predatory diving beetles: a comparison of surface and subterranean photic niches. Royal Society Open Science 2:1–12.

Velarde, R. A., C. D. Sauer, K. K. O. Walden, S. E. Fahrbach, and H. M. Robertson. 2005. Pteropsin: A vertebrate-like non-visual opsin expressed in the honey bee brain. Insect Biochemistry and Molecular Biology 35:1367–1377.

Vogelmann, T. C. 1993. Plant tissue optics. Annual Review of Plant Physiology and Plant Molecular Biology 44:231–251.

Wang, B., J. H. Xiao, S. N. Bian, L. M. Niu, R. W. Murphy, and D. W. Huang. 2013. Evolution and expression plasticity of opsin genes in a fig pollinator, *Ceratosolen solmsi*. PLoS ONE 8:1–11.

Wickham, H. 2009. ggplot2: Elegant graphics for data analysis. Springer-Verlag New York.

Xiang, Y., Q. Yuan, N. Vogt, L. L. Looger, L. Y. Jan, and Y. N. Jan. 2010. Light-avoidance-mediating photoreceptors tile the *Drosophila* larval body wall. Nature 468:921–926.

CHAPTER 2 Tables

Table 1. Summary of primers designed for this study, including melt temperature, product size and nucleotide primer sequence.

Target	Direction	Melt temp (Tm, °C)	Product size (bp)	Primer sequence
LW opsin	Forward	58.7	140	AATCCGATAGTTTACGGCATCAG
LW opsin	Reverse	62.6	140	TTTTCGTCGGCAACATTGGTCAC
UV opsin	Forward	61.3	140	TTTGCGACTTTATGATGATGCTGAAAG
UV opsin	Reverse	61.1	140	GTTCGTCATTCCAGCCCCTATTC
RPS3	Forward	60.4	155	TCGAAGAAACGCAAATTCGTCG
RPS3	Reverse	59.7	155	GTCGCCATAATAATGATCTCTGTACG
b-Actin	Forward	59.8	139	AGGAGCACCCAGTCCTTCT
b-Actin	Reverse	60.5	139	GAAGCGTACAGGGAGAGCAC

Table 2. Summary of *D. ponderosae* annotated opsin nucleotide and protein sequences (NCBI Resource Coordinators 2017).

Dendroctonus ponderosae sequence	Whole-genome shotgun contig	Annotated mRNA sequence	Gene ID	Annotated protein sequence
Long wavelength opsin	APGK01034126.1	XM_019902121.1	LOC109 536053	XP_019757680.1
Ultraviolet opsin	APGK01026615.1	XM_019900023.1	LOC109 534366	XP_019755582.1

Table 3. Summary of qPCR primer efficiencies, calculated from standard curves spanning six orders of magnitude, and melt curve peaks for the long wavelength opsin (LW), ultraviolet opsin (UV, no primer efficiency available), and reference (RPS3 and bAct) gene products.

Primer pair	Target	Primer pair efficiencies (%)	Primer pair R^2	Melt curve peak (°C)
LW	long wavelength opsin	101.7%	0.993	87.5 (87.0, 88.0)
UV	ultraviolet opsin	NA	NA	85.5 (84.5)
RPS3	ribosomal protein subunit 3	104.0%	0.999	83.0 (82.5, 83.5)
bAct	beta-actin	105.2%	0.997	84.5 (85.0, 85.5)

Table 4. Table summarizing the transcription status (+/- = present/absent, NA = not assessed) of the long wavelength and ultraviolet opsins across mountain pine beetle life stages [egg (n = 2), L1-4 = first to fourth instar larva, P = pupa, TA = teneral adult, EA = emerged adult (n = 4)].

Life stage	egg	L1	L2	L3	L4	P	TA	EA
LW opsin expression	+	+	+	+	+	+	+	+
UV opsin expression	NA	NA	NA	NA	-	+	+	+

CHAPTER 2 Figures

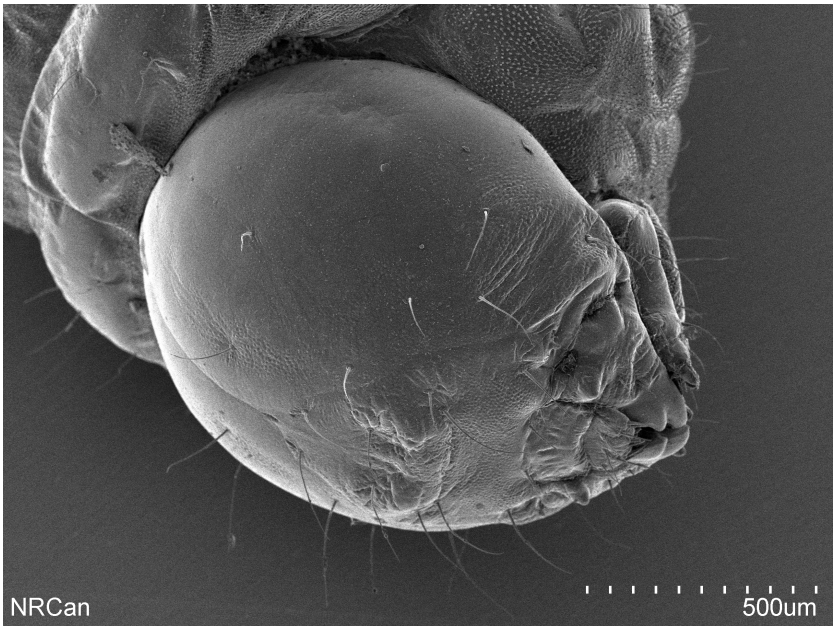


Figure 1. SEM of a mountain pine beetle larva showing the lack of exterior photoreceptor organs (Wertman 2016).

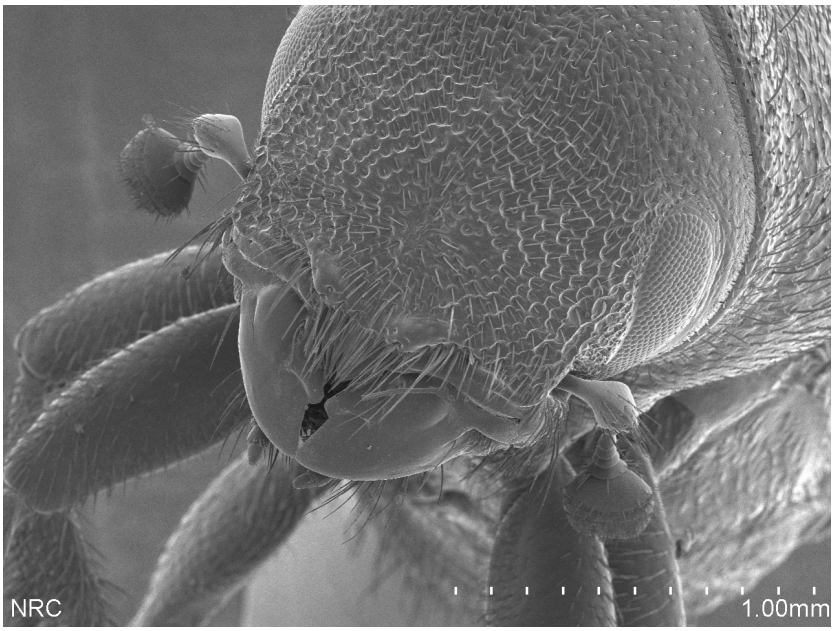


Figure 2. SEM of an adult mountain pine beetle illustrating the apparent absence of ocelli (Wertman 2013).

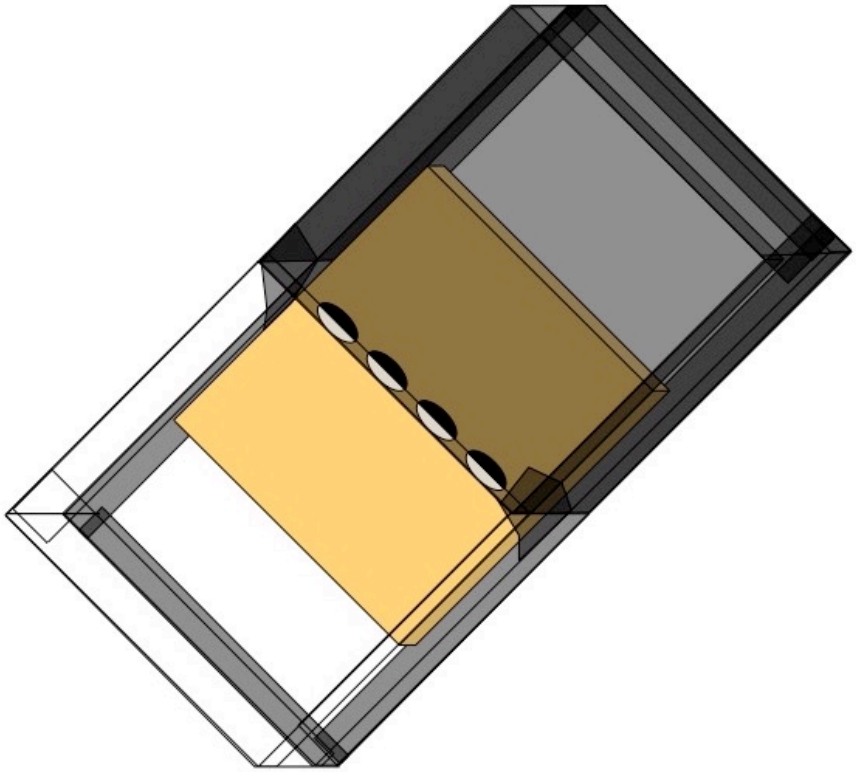


Figure 3. Diagram illustrating the layering of phloem sandwich components, minus temperature probes, larvae, clips and elastic bands. Sandwiches were constructed from base and treatment glass panes, shore pine phloem squares (taupe), clear packing tape, black duct and masking tape (transparent black). Each finished unit exposed one half of each phloem punch to light (white) and the other to dark (black).

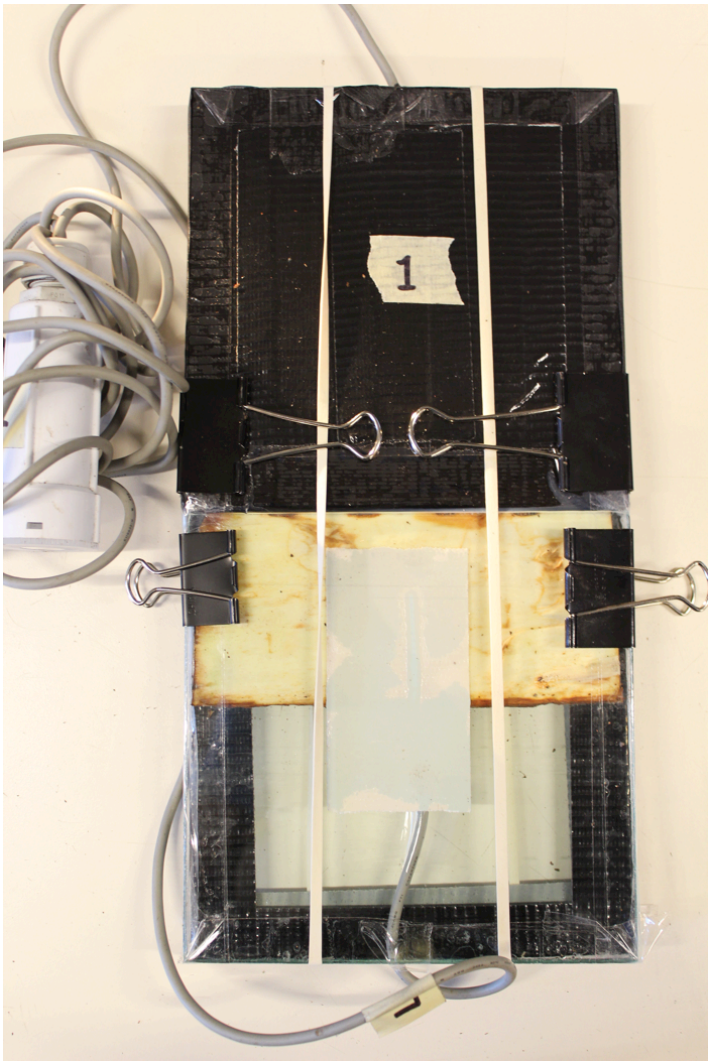


Figure 4. Photograph of a completed phloem sandwich containing temperature probes.

Figure 5. Image showing the placement of a temperature probe within the phloem tissue.

Figure 6. Phloem sandwiches (eight units = one run) arranged in a grid pattern within a white foam core frame, with a bank of two broad-spectrum fluroescent lights suspendend 33.5cm above to produce a maximum light intensity of 4740 lux.

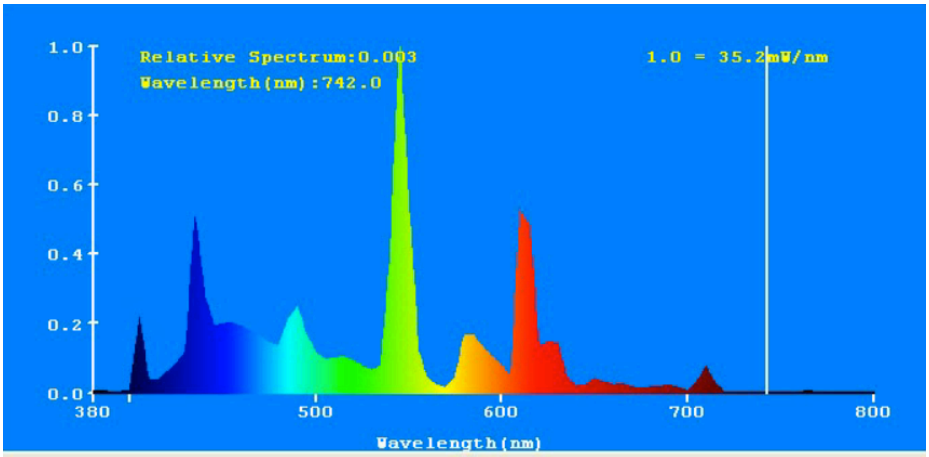


Figure 7. Light spectra profile for the broad-spectrum (6400K) fluorescent lights used for phototaxis experimentation, in mW/nm across wavelengths (nm) (SunBlaster Lighting 2016).

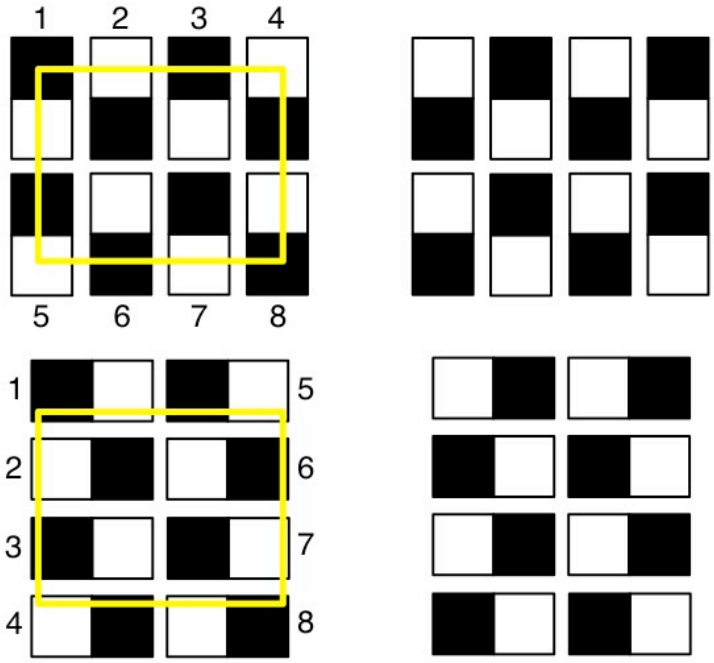


Figure 8. Diagram depicting the four different configurations of phloem sandwich units beneath the light panel (yellow outline) that were represented evenly across phototaxis experiment and control runs.

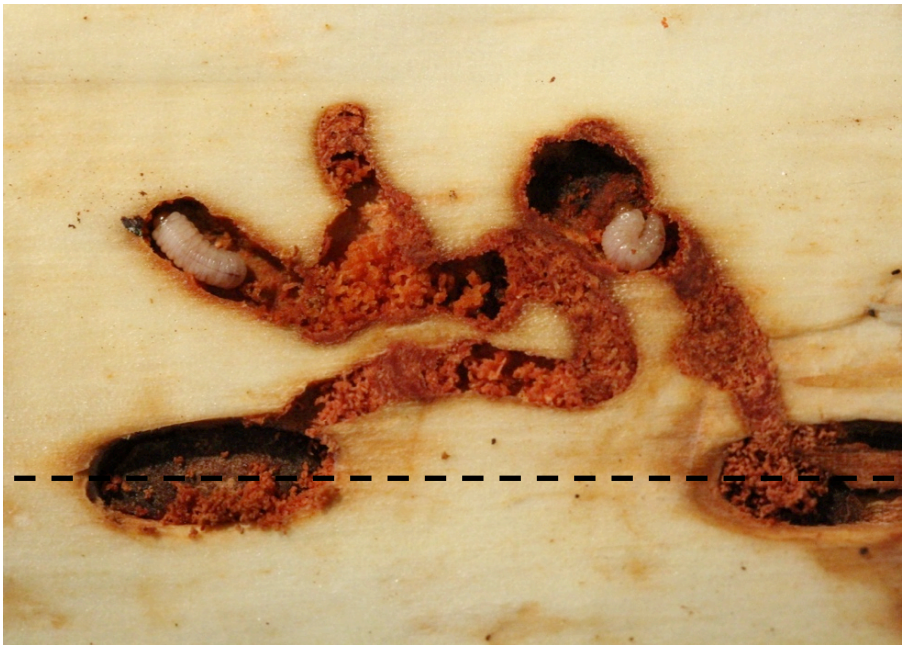


Figure 9. Photograph of two experimental *D. ponderosae* larvae after dissection of a phloem sandwich. These larvae mined beneath the dark panel and were scored as having chosen dark. The dashed line shows the position of the light-dark panel interface in the intact unit.

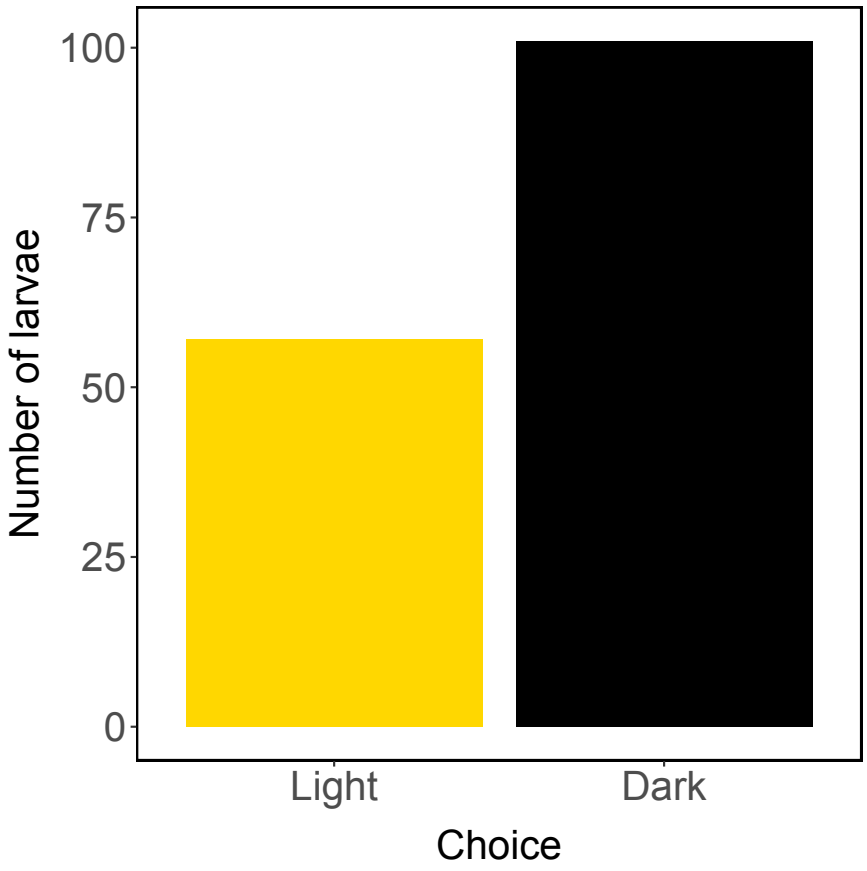


Figure 10. Counts of 158 insects by light preference across eight phototaxis experiment runs (light n = 57, dark n = 101). A chi-square analysis reveals a significant effect of light on *D. ponderosae* larval choice ($\chi^2_1 = 8.8327$: $p < 0.01$). An expected choice ratio was obtained from 71 control larvae assayed in the absence of light (34:37 L:D).

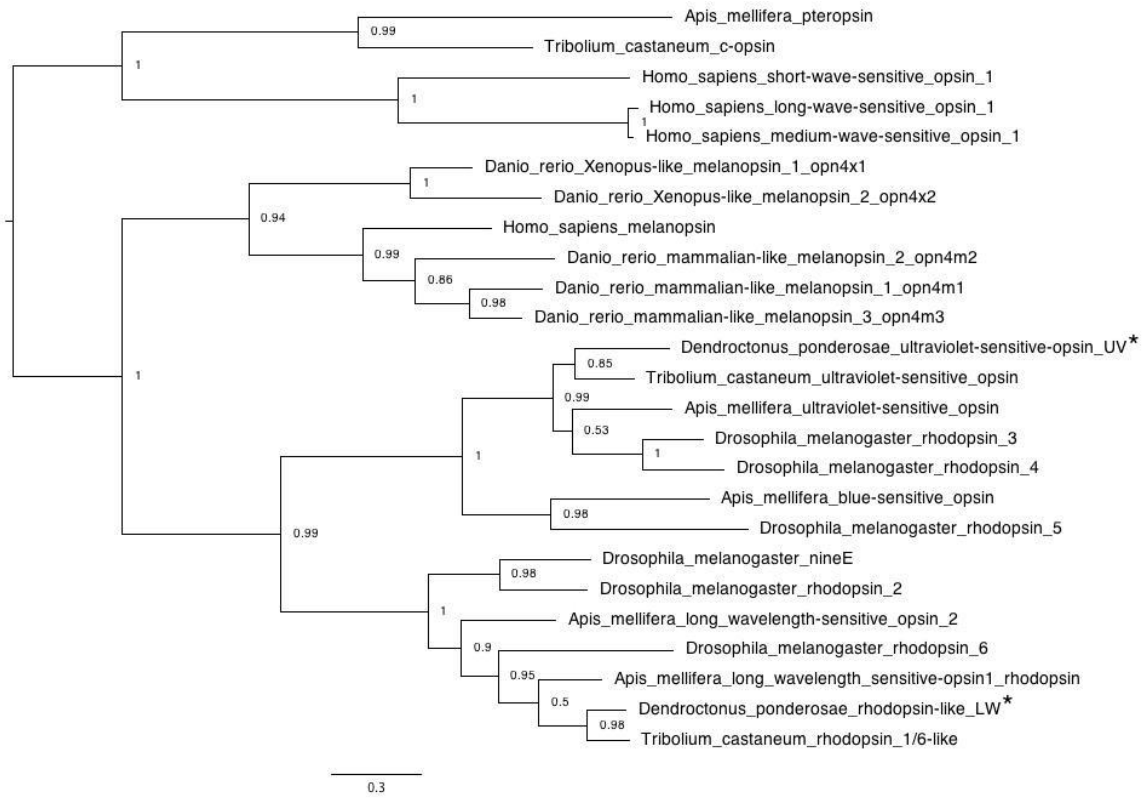


Figure 11. Amino acid phylogeny of *Dendroctonus ponderosae* visual opsins (long wavelength and ultraviolet-sensitive) aligned with *Tribolium castaneum, Apis mellifera, Drosophila melanogaster,* (select) *Danio rerio and Homo sapiens* Opn4 R-type (Provencio et al. 1998, 2000) and C-type opsin sequences. The Opn4 R-type opsin clade (lower) includes both insect visual opsins and vertebrate non-visual melanopsins, while the C-type opsin clade (above) includes vertebrate visual and insect non-visual opsins. Mountain pine beetle sequences are highlighted with an asterisk

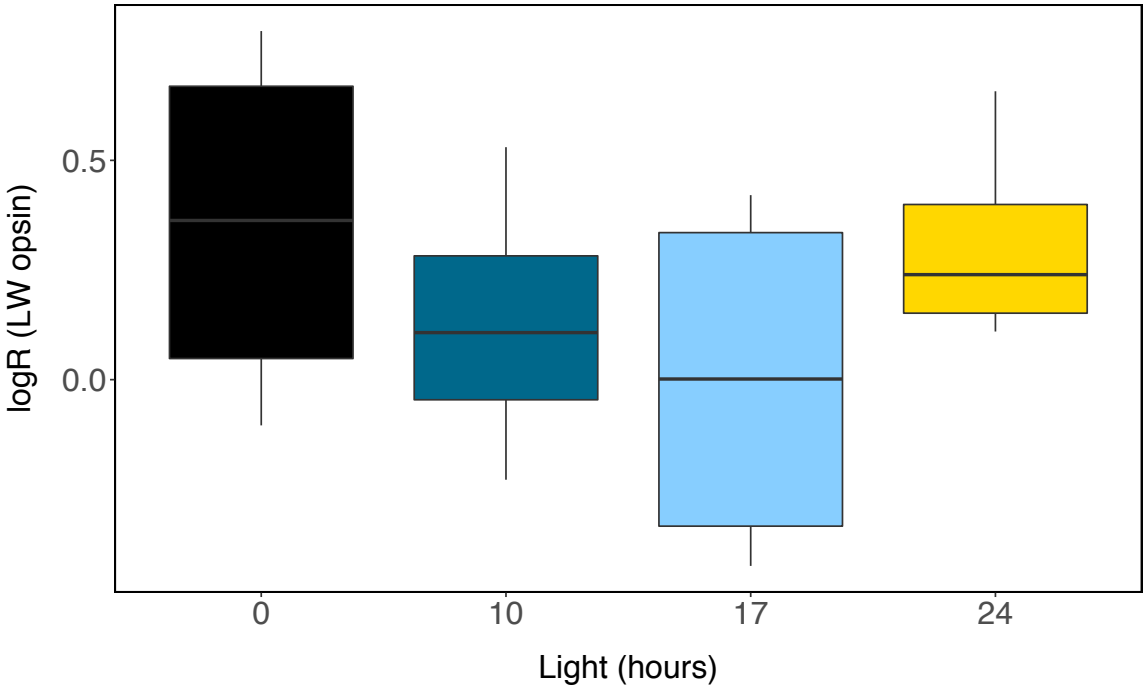


Figure 12. Boxplot for comparison of fold-change long wavelength opsin expression (logR, RPS3) in fourth instar *D. ponderosae* larvae (overall n = 16, n = 4 per treatment) across photoperiods (ANOVA: $F_{3,12} = 0.826$: $p = 0.51$). Larvae were held for two weeks at treatment photoperiods prior to sampling and were collected during the photophase.

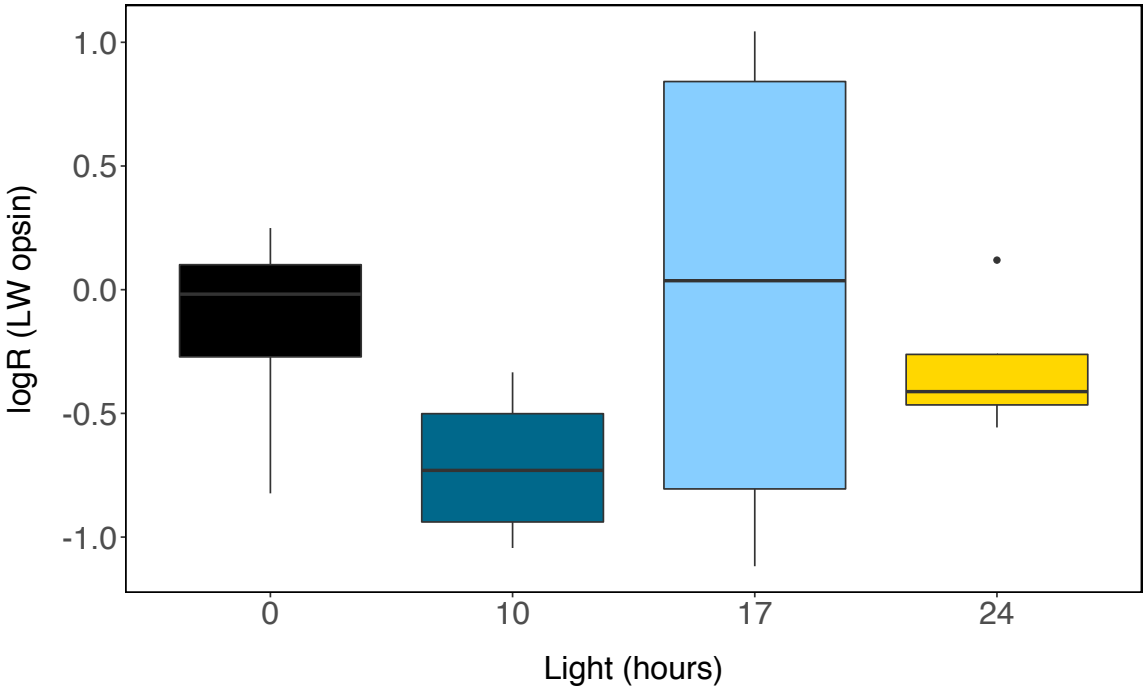


Figure 13. Boxplot for comparison of fold-change long wavelength opsin expression (logR, bAct) in fourth instar *D. ponderosae* larvae (overall n = 16, n = 4 per treatment) across photoperiods (ANOVA: $F_{3,12} = 0.958$: $p = 0.44$). Larvae were held for two weeks at treatment photoperiods prior to sampling and were collected during the photophase.

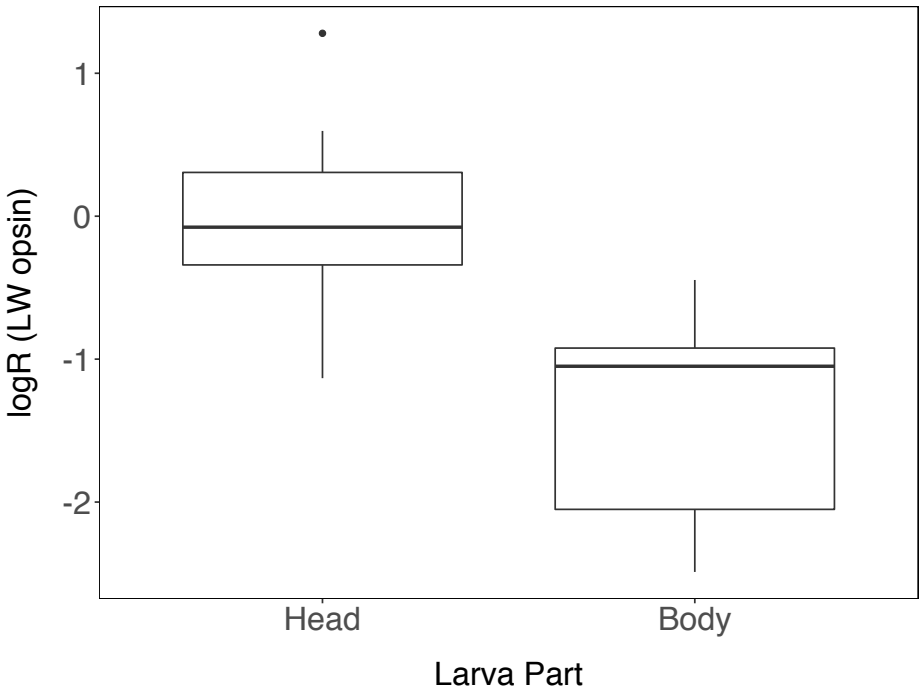


Figure 14. Boxplot of logR values for long wavelength opsin expression in fourth instar larval heads versus bodies (RPS3 reference gene) in *D. ponderosae* (n = 7 larvae). Analysis reveals a significant difference in transcript level between body parts (paired t-test: $t_6 = 2.709$: $p < 0.05$), with expression lower in the body than the head.

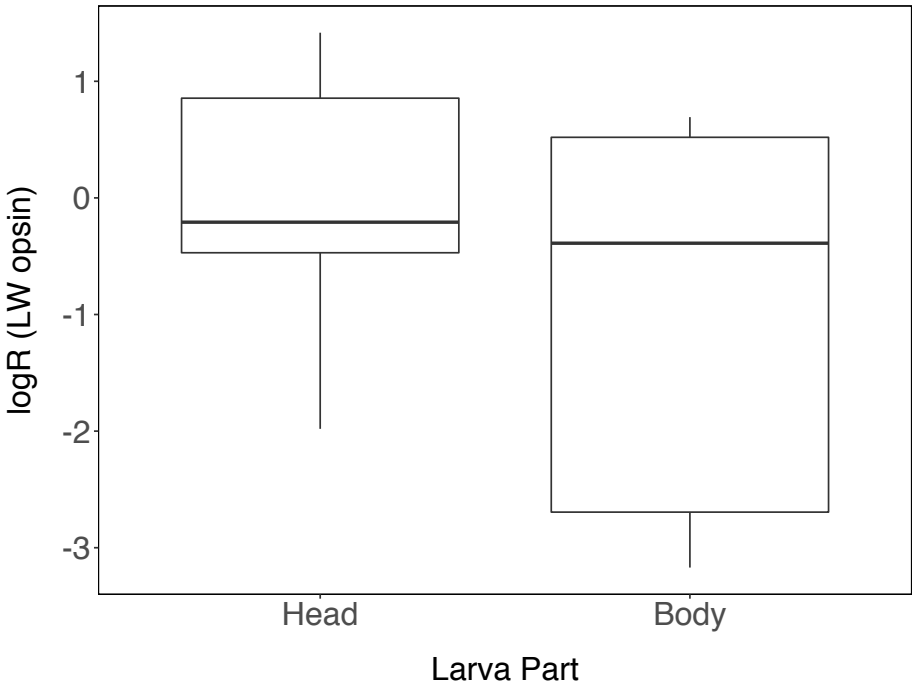


Figure 15. Boxplot of logR values for long wavelength opsin expression in fourth instar larval heads versus bodies (bAct reference gene) in *D. ponderosae* (n = 7 larvae). Analysis reveals a significant difference in transcript level between body parts (paired t-test: $t_6 = 2.9126$: $p < 0.05$), indicating lower expression in the body than the head.

CHAPTER 2 Appendix

Appendix Table 1. *D. ponderosae* samples from development experiments A and B (see CHAPTER 3) used for opsin gene expression analysis in this study (four sets of reactions: 1 = long wavelength opsin expression across photoperiods in larvae, 2 = head versus body long wavelength opsin expression in larvae, 3 = long wavelength opsin transcript presence/absence across life stages, 4 = ultraviolet opsin transcript presence/absence across life stages). L1-4 = first to fourth instar larva, P = pupa, TA = teneral adult, EA = emerged adult.

Reaction set	Experiment source	Photoperiod (L:D h)	Life stage	Replicates
1	A	0:24	L4	4
1	A	10:14	L4	4
1	A	17:7	L4	4
1	A	24:0	L4	4
2	A	17:7	L4	3
2	A	10:14	L4	4
3	B	17:7	egg	2
3	B	17:7	L1	4
3	B	17:7	L2	4
3	B	17:7	L3	3
3,4	A	17:7	L4	4
3,4	A	17:7	P	4
3,4	A	17:7	TA	4
3,4	A	17:7	EA	4

Appendix Table 2. List of *Dendroctonus ponderosae, Tribolium castaneum, Apis mellifera, Drosophila melanogaster, Danio rerio and Homo sapiens* amino acid opsin sequences that were used to construct a phylogenetic tree.

Species	**Phylogeny annotation**	**NCBI annotation**	**NCBI accession number**
Dendroctonus ponderosae	rhodopsin-like (LW opsin)	PREDICTED: rhodopsin-like	XP_019757680.1
Dendroctonus ponderosae	ultraviolet-sensitive-opsin (UV opsin)	PREDICTED: opsin, ultraviolet-sensitive-like	XP_019755582.1
Tribolium castaneum	rhodopsin 1/6-like	rhodopsin 1/6-like	NP_001155991.1
Tribolium castaneum	ultraviolet-sensitive opsin	PREDICTED: opsin, ultraviolet-sensitive isoform X1	XP_970344.2
Tribolium castaneum	c-opsin	c-opsin	NP_001138950.1
Apis mellifera	long wavelength sensitive-opsin 1 (rhodopsin)	rhodopsin, long-wavelength	NP_001011639.2
Apis mellifera	long wavelength-sensitive opsin 2	long wavelength sensitive opsin 2	NP_001071293.1
Apis mellifera	ultraviolet-sensitive opsin	opsin, ultraviolet-sensitive	NP_001011605.1
Apis mellifera	blue-sensitive opsin	opsin, blue-sensitive	NP_001011606.1
Apis mellifera	pteropsin	pteropsin	NP_001035057.1
Drosophila melanogaster	nineE	neither inactivation nor afterpotential E	NP_524407.1
Drosophila melanogaster	rhodopsin 2	psin Rh2	AAA28734.1
Drosophila melanogaster	rhodopsin 3	opsin Rh3	AAA28854.1
Drosophila melanogaster	rhodopsin 4	Rh4 opsin	AAA28856.1
Drosophila melanogaster	rhodopsin 5	rhodopsin 5	AAC47426.1
Drosophila melanogaster	rhodopsin 6	RecName: Full=Opsin Rh6; AltName: Full=Rhodopsin Rh6, long-wavelength	O01668.1
Danio rerio	Xenopus-like melanopsin 1 (opn4x1)	Xenopus-like melanopsin-1	ALG92571.1
Danio rerio	Xenopus-like melanopsin 2 (opn4x2)	Xenopus-like melanopsin-2	ALG92572.1
Danio rerio	mammalian-like	mammalian-like	ALG92568.1

Danio rerio	melanopsin 1 (opn4m1) mammalian-like	melanopsin-1 mammalian-like	ALG92569.1
Danio rerio	melanopsin 2 (opn4m2) mammalian-like	melanopsin-2 mammalian-like	ALG92570.1
Homo sapiens	melanopsin 3 (opn4m3) long-wave-sensitive opsin 1	melanopsin-3 long-wave-sensitive opsin 1	NP_064445.2
Homo sapiens	medium-wave-sensitive opsin 1	medium-wave-sensitive opsin 1	NP_001041646.1
Homo sapiens	short-wave-sensitive opsin 1	short-wave-sensitive opsin 1	NP_001699.1
Homo sapiens	melanopsin	melanopsin	AAF24978.1

Appendix Table 3. Summary of *D. ponderosae* reference genes used for long wavelength opsin expression normalization in this study, and the homologous genes in *T. castaneum*. NCBI accession numbers (*T. castaneum* protein sequences, *D. ponderosae* whole-genome shotgun contig nucleotide sequences encoding reference proteins) and annotations included (* = identified by this study).

Species	**Annotation**	**NCBI reference sequence**
Dendroctonus ponderosae	ribosomal protein subunit 3*	APGK01036313.1
Dendroctonus ponderosae	beta-actin*	APGL01029974.1
Tribolium castaneum	ribosomal protein S3	NP_001165863.1
Tribolium castaneum	actin related protein 1	NP_001165844.1

Appendix Table 4. ANOVA output table for the comparison of logR values in fourth instar *D. ponderosae* larvae (overall n = 16, n = 4 per treatment) across photoperiods (RPS3).

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Light	3	0.3253	0.1084	0.826	0.505
Residuals	12	1.5757	0.1313		

Appendix Table 5. ANOVA output table for the comparison of logR values in fourth instar *D. ponderosae* larvae (overall n = 16, n = 4 per treatment) across photoperiods (bAct).

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Light	3	1.118	0.3728	0.958	0.444
Residuals	12	4.669	0.3890		

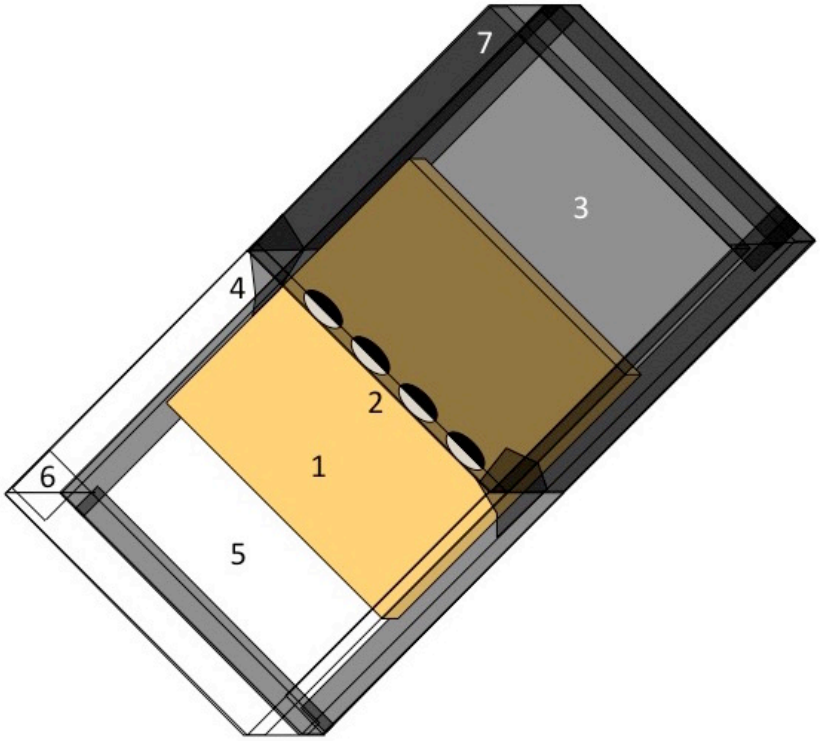
See attached files:

Chapter2Appendix_Phototaxis.xlsx

Appendix Table 6. Phototaxis experiment data.
Appendix Table 7. Phototaxis control data.
Appendix Table 8. Phototaxis experiment temperature data.
Appendix Table 9. Phototaxis control temperature data.

Chapter2Appendix_qPCR.xlsx

Appendix Table 10. qPCR long wavelength opsin (LW) raw Cqs across life stages.
Appendix Table 11. qPCR reference gene (RPS3) raw Cqs across life stages.
Appendix Table 12. qPCR reference gene (bAct) raw Cqs across life stages.
Appendix Table 13. qPCR ultraviolet opsin (UV) raw Cqs across life stages.
Appendix Table 14. qPCR long wavelength opsin (LW) raw Cqs for L4s across photoperiods.
Appendix Table 15. qPCR reference gene (RPS3) raw Cqs for L4s across photoperiods.
Appendix Table 16. qPCR reference gene (bAct) raw Cqs for L4s across photoperiods.
Appendix Table 17. qPCR long wavelength opsin (LW) raw Cqs for L4s head/body.
Appendix Table 18. qPCR reference gene (RPS3) raw Cqs for L4s head/body.
Appendix Table 19. qPCR reference gene (bAct) raw Cqs for L4s head/body.



Appendix Figure 1. Diagram illustrating the assembly order of phloem sandwich components, minus temperature probes, larvae, clips and elastic bands. 1) A phloem square was peeled away from the sapwood and placed phloem side up in the centre of a large pane of glass (15cm X 30cm) edged with black duct tape; 2) four 1.2cm X 0.5cm oval-shaped punches (for larvae) were made horizontally along the centre of the square; 3) the dark panel was secured to the sandwich using clear packing tape, ensuring even division of the punches; 4) black masking tape was placed along the outside edges of the phloem at the panel interface to block light from entering the dark treatment via the transparent pane; 5) the transparent panel was placed directly against the inside edge of the dark panel and attached with packing tape; 6) the entire sandwich was then secured tightly with packing tape, including all edges and along the panel interface; 7) black masking tape was added to the outer edge of the dark panel to seal the treatment from light; 8) (not shown) two elastics were placed lengthwise over the sandwich, followed by four 0.75” alligator clips (two per side) for tightening.

CHAPTER 3:

Effects of photoperiod on development and emergence rates in the mountain pine beetle (*Dendroctonus ponderosae*)

INTRODUCTION

"…photoperiodism cannot be disregarded when evaluating the mechanisms underlying life-historical events in any animal living at temperate and polar latitudes."

- Bradshaw and Holzapfel 2007

The seasonal phenology of insects living in high latitude temperate regions is, in general, highly mediated by photoperiod (Bradshaw and Holzapfel 2007). The effects of light and photoperiodism on life cycle coordination in cryptic bark beetles and cerambycids developing within subcortical plant tissues are poorly investigated, with only a handful of systems explored to date (Shintani et al. 1996, Doležal and Sehnal 2007, Shintani 2011). The mountain pine beetle *Dendroctonus ponderosae Hopkins* (Coleoptera: Curculionidae, Scolytinae), a severe pest of mature pine in north western North America, possesses temperature-dependent mechanisms for life cycle synchronization and overwinter survival (Safranyik 1978, Bentz et al. 1991, Bentz et al. 2013). Despite the highly coordinated seasonal phenology of *D. ponderosae* across broad latitudes (Bentz et al. 1991, Bentz et al. 2013), an effect of photoperiod on immature development and emergence rates has not been thoroughly investigated in this species. I contend that photoperiodism cannot be excluded as a mechanism contributing to *D. ponderosae* life cycle synchronicity without thorough experimental analysis.

Insect photoperiodism and circadian regulation

The earth's rotation about its axis and around the sun generates daily 24 hour and annual 12 month rhythms characterized by shifting day lengths, producing predictable cues for seasonal anticipation (Bradshaw and Holzapfel 2007). Photoperiodism is the mechanism by which insects and other organisms are able to perceive and prepare for seasonal change, functioning in the regulation and synchronization of physiological processes that

are essential to the life cycle in future time or space. Photoperiod changes predictably with latitude, with shorter winter day lengths and steeper phenological effects proceeding northwards from the equator, and thus preparation for seasonal events is vital for survival in temperate regions (Bradshaw and Holzapfel 2007, Hut et al. 2013). Although effects of photoperiod on development, dormancy and circadian regulation are widely described for beetles and other insects (Doležal et al. 2007, Lopatina et al. 2011, Saunders 2012), there have been very few investigations into photoperiodic effects on immature insects inhabiting subcortical tree tissues (Shintani et al. 1996, Doležal and Sehnal 2007, Shintani 2011).

All eukaryotes are thought to possess an endogenous circadian clock with an approximately 24 hour cycle (Emerson et al. 2009). The insect circadian clock is proposed to consist of an inherent biological rhythm that can be entrained via day length, and a molecular mechanism for photoperiodic time measurement (Saunders 2002, Emerson et al. 2009, Bradshaw and Holzapfel 2010). The internal circadian clock functions in daily environmental tracking, entraining to dusk and dawn using light and temperature cues, while the photoperiodic timer functions in seasonal anticipation and physiological programming for life history events including development, dormancy, reproduction and migration (Bradshaw and Holzapfel 2010). Although the functional link between the photoperiodic and circadian timers in insects is not fully resolved, it appears to involve clock genes (Sandrelli et al. 2007, Tauber et al. 2007, Ikeno et al. 2010).

Insect dormancy

Insects engage in a diverse range of species and life stage-specific dormancies, metabolic suppression that functions to reduce energy demands during unfavourable conditions, optimize winter survival, and facilitate seasonal synchronicity (Koštál 2006, Belozerov 2009). Dormancy and diapause, a type of dormancy, are often found in temperate regions with adverse seasonal conditions. The perception of shortening day lengths induces diapause in many insect species, while long days characteristic of summer usually promote development (Bradshaw and Holzapfel 2001, Saunders 2012).

Koštál (2006) and Belozerov (2009) have attempted to clarify the difficult distinctions among diapause and types of non-diapause dormancy, which differ along a continuum of physiological responses to environmental variables. Diapause may be defined as the programmed physiological re-routing of development into a dormant state in response to environmental thresholds, while non-diapause dormancy is described generally as metabolic suppression in direct response to environmental factors (Koštál 2006, Belozerov 2009). Diapause, which may be facultative or obligate, is induced prior to the onset of adverse conditions. The effects of non-diapause dormancy on insect life cycle regulation are not well understood, and the lack of reliable diagnostics to characterize dormancy in study organisms is limiting (Belozerov 2009).

The mountain pine beetle

The spread of the mountain pine beetle *Dendroctonus ponderosae* Hopkins (Coleoptera: Curculionidae, Scolytinae) from its native range is the greatest threat currently facing Canada's boreal forest (Safranyik and Carroll, 2006; Safranyik et al. 2010). The north and eastward range expansion of *D. ponderosae*, in part facilitated by climate change, is exemplified by the breach of the presumed historical geographic barrier to the boreal forest, the Rocky Mountains (Safranyik et al. 2010). Most populations of mountain pine beetle are univoltine, although semivoltine life cycles may be found at cool northern latitudes and high elevations (Amman 1973, Bentz et al. 1991, 2013).

Life cycle and emergence synchronicity in *D. ponderosae* optimizes the success of mass attacks on host trees and contributes to the establishment of epidemic populations (Bentz et al. 1991). In temperate univoltine populations, adult beetles emerge from their brood trees and disperse to colonize new hosts in late summer, laying eggs within the phloem layer (Safranyik and Carroll 2006). A new generation of larvae hatches and matures, overwinters in the fourth instar, then resumes development and pupates in the late spring/early summer. Timing of adult emergence is strongly influenced by development rate, which is primarily determined by temperature (Bentz et al. 2013). Genetic and phenotypic variation in the developmental response of *D. ponderosae* to temperature helps to maintain consistent phenology across its climatically diverse range.

Mountain pine beetle have diapause-independent adaptations for univoltine life cycle coordination and overwinter survival across its broad latitudinal range (Bentz et al. 1991), which extends from northern Mexico to northern British Columbia (Safranyik and Carroll 2006). Life stage specific temperature thresholds for development function to synchronize life cycles across climates and seasonal environments, ensuring that the cold-hardy fourth instar larval stage is reached before entry into winter dormancy (Safranyik and Carroll 2006). *D. ponderosae* exhibits genetic variability in development rate across its range, with northern populations completing development under fewer degree days than southern populations, and effectively compensating for cooler temperatures found at high latitudes (Bentz et al. 2011, 2013, Bracewell et al. 2013). Early instar larvae have lower developmental temperature thresholds than late instar larvae, functioning to prevent pupation before the arrival of winter (Bentz et al. 1991, 2013). Developmental coordination is maintained by temperature thresholds by allowing younger larvae to catch up with fourth instars before the arrival of winter, therefore maximizing fitness in unpredictable environments by reducing cold-related mortality and synchronizing adult development.

Dormancy in bark beetles

Facultative adult reproductive diapause in the European spruce bark beetle (*Ips typographus*) is well established (Doležal and Sehnal 2007, Jönsson et al. 2011), while facultative diapause is documented for spruce beetle (*Dendroctonus rufipennis*) prepupae (Hansen et al. 2011) and suggested for *D. ponderosae* adults (Lester and Irwin 2012). *I. typographus* can produce up to three generations during warm summers at certain latitudes, until the shortening day lengths characteristic of late summer induce an adult diapause (Doležal and Sehnal 2007). Diapause must be initiated when the insect anticipates that winter will arrive before a new generation can complete development, as the insects are most likely to survive winter temperatures in the cold hardy adult stage (Jönsson et al. 2011). Diapause incidence in *I. typographus* is positively associated with latitude and decreasing photoperiod, with longer day lengths inducing diapause in the northern extent of its range (Doležal and Sehnal 2007, Schroeder and Dalin 2016).

The spruce beetle *Dendroctonus rufipennis*, which develops within and emerges from the phloem layer of spruce tree (*Picea* spp.) hosts, has a one to three year life cycle depending on environmental temperature and the induction of facultative diapause (Hansen et al. 2011). Pre-pupal winter diapause is found in semivoltine populations and is induced if an insufficient number of degree days have been accumulated prior to autumn, as pupae are not cold tolerant and suffer high winter mortality. Hansen et al. (2011) found the induction of prepupal diapause in *D. rufipennis* to occur independently of photoperiod when reared from the second or third instar under two photoperiods (long and short day) and at two tested temperatures.

Mountain pine beetles overwintering as adults, like late instar larvae, suppress metabolism in response to cold temperatures (Lester and Irwin 2012). Lester and Irwin (2012) report possible evidence of facultative diapause in mountain pine beetle adults, citing trends in metabolic activity, decreased supercooling point and lack of response to temperature variation as evidence. While it is apparent than mountain pine beetle overwintering may be defined as dormancy, it is not yet supported that the beetles undergo the profound physiological interruption of development that is characteristic of diapause (Koštál 2006), nor that the attribution of facultative diapause to *D. ponderosae* is supported by the data presented by Lester and Irwin (2012).

Framework for study

The photoperiodic and seasonal adaptations of insects can be difficult to identify, but with the earlier arrival of spring and extended growing seasons as consequences of climate change (Bradshaw and Holzapfel 2001), it is increasingly important to tease apart the effects of light from those of temperature on the development and life histories of pest species. Effects of photoperiod on development and growth rate, diapause, body weight and instar number are widely described for insects (Lopatina et al. 2011). Exposure of immature beetles to varying photoperiods is known to influence adult reproductive diapause induction in *I. typographus* (Doležal and Sehnal 2007), while photoperiod has also been found to affect yellow-spotted longicorn beetle *Psacothea hilaris* (Shintani et al. 1996) and chrysanthemum longicorn beetle *Phytoecia rufiventris* (Shintani 2011)

diapause, number of larval instars and rate of development. There is also evidence for an endogenous circadian emergence rhythm in *D. ponderosae* that I suggest may result from photoperiodic entrainment of the immature insects (Watson 1970, Billings and Gara 1975, Safranyik 1978, Safranyik et al. 1989).

Given the seemingly ubiquitous effects of photoperiod and light on insect life histories, I hypothesize that some photoperiodism and circadian regulation by light is likely maintained in mountain pine beetles developing within the subcortical tissues of host trees. I previously identified *D. ponderosae* larval photosensitivity in a behavioural experiment, as well as transcription of a long wavelength opsin ortholog across all life stages, including eggs and eyeless larvae; these results indicate that the beetle likely possesses the capacity for photoreception continuously throughout its life cycle (see CHAPTER 2). The objectives of this study are to 1) determine whether *D. ponderosae* development and/or emergence rate vary across photoperiods (experiments A and B, reared from field-collected mature larvae), and 2) to assess the development and emergence rates of beetles reared from the egg stage in the complete absence of light (experiment B).

METHODS

Development experiment A

Photoperiod treatments

To test for an effect of photoperiod on the development and emergence rates of *D. ponderosae*, I reared insects at 18°C under four photoperiods (light:dark): 0:24, 10:14, 17:7, 24:0 (see METHODS: Experimental treatment selection). Insect development was sampled periodically (see Insect source and sampling, below). Photoperiod treatments were applied in walk-in growth chambers with the exception of 0:24, which was executed in a reach-in growth chamber with an exterior blackout curtain. Experimental chambers were equipped with broad-spectrum fluorescent lighting (Figure 1., Philips F48T8/TL841/HO 4100K 44 Watt Hg 2L bulbs, Alto Collection, USA). Light intensity (illuminance, lux) was assessed in each chamber using a Digital Light Meter (DLM2, UEi Test Instruments, Richmond BC) (overall experiment means: 10:14 2110 lux, 17:7 2550 lux, 24:0 2460 lux).

Two or three two-channel temperature loggers [HOBO Pro v2 2X External Temperature Data Logger (Part # U23-003), Onset Computer Corporation, Bourne MA] were used to record the temperature in each chamber every five minutes. The manufacturer reports the logger channel accuracy as ± 0.21°C and the resolution as ± 0.02°C, while my independent calculations reveal a precision estimate of ± 0.050°C (from two sets of sixteen measurements held constantly at ~19°C). Temperature in each chamber was measured for the duration of the experiment (approximately 17 weeks); however, equipment failure led to loss of temperature data from each logger for periods ranging from 0.5 to 12 weeks (average five weeks). The mean (SD) temperature for each chamber was calculated using the available files and were as follows: 0:24, 18.0°C (0.51); 10:14, 17.8°C (0.29); 17:7, 17.9°C (0.21); 24:0, 17.9°C (0.27) (HOBOware Pro Version 3.7.4, Onset Computer Corporation, Bourne MA). Degree days were calculated using the mean temperature measured in each chamber (see Data analysis, below).

Insect source and sampling

Two mountain pine beetle infested lodgepole pine trees that were felled and cut into bolts 30 to 40cm long on 24 September 2015 near *Musreau* Lake outside Grande Prairie, Alberta (GPS coordinates: tree 2 N54.54737° W118.71753°, tree 5 N54.56583° W118.71144°). The trees were attacked by mountain pine beetle in the summer of 2015 and the developing brood were primarily mid instar larvae at this time. Bolts were transported to the Pacific Forestry Centre (Victoria, British Columbia) and the ends were sealed with paraffin wax to prevent excessive moisture loss. The bolts were then placed in a walk-in growth chamber at 15°C 10:14 for 23 days to allow the brood to develop through to late instar larvae before initiating the experiment. The 10:14 photoperiod was selected to represent the field site in late fall (see METHODS: Experimental treatment selection), while the 15°C rearing temperature permitted younger larvae to catch up to mature instars. Insect development was periodically monitored by removing pieces of bark from several bolts and observing the life stages present.
Once most of the developing brood were observed to be late instar larvae, bolts were given a short cold period in an environmental chamber (Envirotronics ENDH1000 990-liter temperature test chamber, Grand Rapids, MI). The bolts were cooled by 2°C a day from a daily mean temperature of 11°C to -5°C. A diurnal variation of ± 7°C about the mean was used and the temperature was held at the daily minimum and maximum for one hour. The change in temperature between the daily minimum and maximum occurred at a constant rate. Following the nine day cooling period, the bolts were placed in a -2°C walk-in freezer for 10.5 days and then in a 5°C walk-in cold room for 14 days before they were moved to one of the four photoperiod treatments at 18°C (day 0).

Two bolts from each of the two trees (four total) were randomly assigned to one of four photoperiod treatments. Insect development was sampled on days 0, 8, 13, 20, 27, 34, 41, 48 and 116/117 (2.5 weeks following emergence completion). Sampling was alternated between bolts from the same tree at each sample time. Proceeding from the base of each bolt, a clean line was chiselled around its circumference to form a sampling ring ~1/5th the height of the bolt. The bark of this ring was peeled to collect all live and dead mountain pine beetle (excluding parent adults, parasitized, predated, hard/black and

wax damaged larvae), and counts recorded for each life stage. Bolts were placed into mesh cages for emergence collection on day 40, stacked by tree (two bolts from the same tree per cage) so that emergence was pooled by tree. Emerged beetles were collected twice weekly (one emergence collection interval = 3 or 4 days) from each treatment chamber.

For a separate study (see CHAPTER 2), select mountain pine beetle samples representative of all available life stages and photoperiods were preserved for opsin gene expression analysis. Healthy looking live insects were grouped by life stage (into Eppendorf Biopur Safe-Lock Microcentrifuge Tubes, 1.5mL) and frozen at -80°C.

Data analysis

To test for an effect of photoperiod on mountain pine beetle development rate, the number of individuals in each life stage was determined for each sample and used to calculate the "proportion larvae" present (the proportion of insects that had not pupated, excluding eggs). This process generated binomial response data to test against the categorical explanatory variables degree day and photoperiod. For analysis of emergence rate in response to photoperiod, the number of degree days required for each individual adult to emerge (recorded in three or four day intervals) were used as dependent count data to test against the independent photoperiod variable. Degree days were calculated using the estimated lower developmental temperature threshold for *D. ponderosae* of 5.6°C (Safranyik 1978) and the mean temperature for each chamber, as stated previously. The mean (SD) numbers of insects per section for either tree were 77 (39.5) and 13 (10.6); data from the second tree were omitted from analysis due to low sample size.

A binomial logistic generalized linear model was used to evaluate proportion larvae (n = 24 observations across six time points) in response to photoperiod (0:24, 10:14, 17:7, 24:0) and degree day. The experiment A proportion larvae dataset used for analysis begins on day 8 (bolts sampled on day 0 were not assigned to photoperiods), and as a result of pooled emergence collections, ends on day 41 prior to emergence initiation (day 48). A Poisson model was implemented to evaluate number of degree days to emergence

(n = 973 beetles) by photoperiod, extending experiment A data analysis to experiment completion. Analyses were performed in RStudio (RStudio Team 2015, R Core Team 2015), and plots were generated using ggplot2 (Wickham 2009).

Development experiment B

Photoperiod treatments

To determine whether day length exposure prior to the third larval instar affects development and/or emergence rates, I reared mountain pine beetle from the egg stage at 18°C under two different photoperiods (0:24 and 17:7). Insects were sampled regularly throughout development (see Insect source and sampling, below). Photoperiod treatments were applied, and environmental variables (temperature, light intensity) monitored, as described for experiment A. Due to human error, bolts at 17:7 (2160 lux) were exposed to three nights of constant light from days 6 to 9. Two two-channel temperature loggers [HOBO Pro v2 2X External Temperature Data Logger (Part # U23-003), Onset Computer Corporation, Bourne MA] recorded the temperature in each chamber throughout the experiment (approximately 22 weeks).

Mean temperatures for either chamber, corresponding to the time frame of each dataset, were obtained from complete series and incorporated into degree day calculations for each respective test (see Data analysis, below). The mean (SD) temperatures were as follows: up to day 54 (last proportion sample for analysis) 0:24, 18.6°C (0.33); 17:7, 18.5°C (0.38); up to day 89 (last observed pupae) 0:24, 18.7°C (0.36); 17:7, 18.6°C (0.44); up to day 142 (last observed emergence) 0:24, 18.8°C (0.40); 17:7, 18.7°C (0.48), and up to day 156 (experiment completion) 0:24, 18.9°C (0.43); and 17:7, 18.8°C (0.60) (HOBOware Pro Version 3.7.4, Onset Computer Corporation, Bourne MA).

Insect source and sampling

To obtain eggs for experimentation, bolts cut from a healthy pine tree were attacked by mountain pine beetle in the laboratory. Eight ~30cm bolts of fresh shore pine were harvested from Jordan River, Vancouver Island (N48.40119° W123.88644°) in late

February 2017, and returned to the Pacific Forestry Centre. The bolt ends were sealed with paraffin wax and moved to a 6.2°C walk-in chamber for short term storage. These bolts were subsequently placed in a climate controlled (~22°C 17:7) sealed room, with an assortment of mountain pine beetle infested lodgepole pine bolts containing teneral and emerging adults (collected at N54.31310° W116.53765°). The insects were allowed to infest the fresh wood under these conditions for nine days, during which time the bolts were periodically rotated and attacks marked by date. The egg-containing bolts were then moved to one of the two experimental treatment chambers at 18°C (day 0).

The infested bolts were randomly assigned to either photoperiod, and to a sampling order within each treatment. Sampling occurred on days 5, 12, 19, 26, 33, 40, 47, 54, 61, 68, 75, 82, 89, and 156 (two weeks following emergence completion). Bolts were also sampled on day 0 to ensure that only eggs were present at experiment commencement. Bolts were sampled in loosely defined vertical sections until a minimum of thirty insects from at least two female galleries were collected [mean (SD) number insects per sample = 45 (6.5)]. Bolts were caged together by treatment on day 54, and emerged adults collected on day 61 and then biweekly (one collection period = 3 or 4 days) until emergence was complete.

Some insects from experiment B were also preserved for opsin gene expression analysis (see CHAPTER 2).

Data analysis

As for experiment A, I used the number of individuals from each life stage to calculate the proportion of larvae present in each sample (n = 10 observations across five time points) for testing against photoperiod (0:24 and 17:7) and degree day in a binomial logistic model. Proportions were calculated from day 26 (egg hatching complete) up until day 54, just prior to the initiation of adult emergence (day 61). Poisson models were used to test degree days to pupation (n = 69 pupae) and emergence (n = 310 beetles) in response to photoperiod. Since the first emergence collection interval was one week as opposed to 3 or 4 days, adult counts from this period were divided in half to reveal the

average number of beetles over two 3.5 day intervals. Degree days were calculated from experiment B temperature series subsets corresponding to specific dataset time frames, as previously discussed. Analyses were performed in RStudio (RStudio Team 2015, R Core Team 2015), and plots produced in ggplot2 (Wickham 2009).

Experimental treatment selection

Experimental rearing temperature and photoperiods for development experiments A and B were chosen based on established developmental temperature thresholds and natural variability in summer/fall day lengths at northern latitudes. I selected an experimental treatment temperature of 18°C to ensure enough heat accumulation for successful development, but near enough to 15°C to capture any effect of photoperiod around the threshold temperature. *D. ponderosae* develops optimally at 23-25°C (Reid and Gates 1970, Safranyik and Whitney 1985, Bentz et al. 1991); although eggs and young larvae can develop at temperatures above 5.6°C, older larval instars appear to require temperatures above 15°C for both development and pupation (Safranyik and Whitney 1985). Emergence, which is restricted below 16°C, should also occur at 18°C despite the fact that *D. ponderosae* generally emerge at temperatures above 20°C (Safranyik and Carroll 2006).

I selected the longest and shortest day length treatments that are biologically relevant to the population of *D. ponderosae* under study, representing periods of summer development (17:7) and overwintering (10:14). Constant light (24:0) and constant dark (0:24) treatments were included as potential positive and negative controls. At N54° in north-central Alberta, the source population of beetles experiences day lengths typical of northern latitudes that are highly seasonal, with long summer and short winter photoperiods. Specifically, the region experiences the longest days in the summer (17:7, mid/late June), day lengths of ~12 hours in early autumn (mid/late September), and shorter days later in the fall (10:14, late October) (NOAA ESRL Sunrise/Sunset Calculator 2017).

I applied a brief cold treatment to my field-collected third and fourth instar larvae in experiment A to simulate exposure to winter temperatures. I did not expose my experiment B beetles that were reared from egg in the laboratory to cold as fourth instar larvae because photoperiodic exposure could not be maintained throughout the cold treatment.

RESULTS

Development experiment A

I did not detect an effect of photoperiod on *D. ponderosae* development rate when reared from field-collected mature larvae (binomial logistic model: $z_{21} = 0.267$: $p = 0.79$); however, degree day was highly significant ($z_{21} = -19.227$: $p < 0.0001$) (Table 1., Figure 2.). The significance of degree day was anticipated due to the strong effects of heat accumulation over time on beetle development. The field-collected insects took an average of 1166 degree days (DD) to complete emergence when reared from mature larvae (0:24 1153 DD, 10:14 1049 DD, 17:7 and 24:0 1230 DD). To achieve 50% emergence, the beetles required 891 DD (0:24 942 DD, 10:14 800 DD, 17:7 935 DD, 24:0 886 DD). The numbers of adult *D. ponderosae* emerging from each treatment were as follows: 0:24 n = 257, 10:14 n = 259, 17:7 n = 250, 24:0 n = 207.

Photoperiod had a significant effect on emergence rate (Poisson model: $z_{971} = -6.682$: $p < 0.0001$) (Table 1.). Insects exhibited steeper emergence peaks in complete darkness and at a short day length than under constant light and long day conditions, where emergence was more gradual and extended over time (Figure 3.).

Development experiment B

I did identify a significant effect of photoperiod on *D. ponderosae* development rate when reared from the egg stage (binomial logistic model: $z_7 = 2.637$: $p < 0.01$); degree day was also significant ($z_7 = -6.605$: $p < 0.0001$) (Table 2.). Development rate was accelerated under continued darkness as compared to the long day (17:7) photoperiod (Figure 4.). The insects took an average of 1702 degree days (DD) to complete emergence when reared from the egg stage (0:24 1544 DD, 17:7 1860 DD), and 50% emergence was reached at an average of 966 DD (0:24 950 DD, 17:7 983 DD). The numbers of beetles emerging from the 0:24 and 17:7 treatments were 114 and 175, respectively.

Analyses revealed significant effects of photoperiod on number of degree days to both pupation (Poisson model: $z_{67} = 16.85$: $p < 0.0001$) and emergence (Poisson model: $z_{308} =$

20.08: $p < 0.0001$) in experiment B (Table 2.). *D. ponderosae* reared from eggs pupated and emerged earlier in complete darkness than under long day lengths (Figures 5. and 6.). The pupation result is consistent with the binomial model, and with the beetles exhibiting steeper emergence under constant darkness (0:24) and protracted emergence under long day (17:7) exposure, the emergence trends parallel experiment A observations.

DISCUSSION

The combined results from development experiments A and B are highly suggestive of a significant effect of photoperiod on *D. ponderosae* emergence activity, as well as indicating an effect of light on immature beetle development rate when reared from the egg stage, but not from third/fourth instars, under constant photoperiod treatments. Mountain pine beetles are able to successfully complete development when reared from the egg stage in complete darkness (experiment B). I detected a significant effect of photoperiod on emergence rate, but no effect of light on the development rate of field-collected third and fourth instar larvae in experiment A. Analyses also reveal effects of photoperiod on overall immature beetle development, pupation and emergence rates when reared from the egg stage under experimental treatment conditions in experiment B. Contrasting development trends in response to photoperiod between experiments A and B indicate that the critical photosensitive life stage(s) in *D. ponderosae* occurs prior to the third larval instar. Emerging adult beetles are sensitive to photoperiod regardless of exposure at the immature stages.

Effects of photoperiod on development rate

This study revealed an effect of photoperiod on development rate when *D. ponderosae* were reared to emergence under experimental conditions from eggs (experiment B), but not when reared from mature (third or fourth instar) field-collected larvae (experiment A). The most logical interpretation of this discrepancy is that the critical photosensitive stage for *D. ponderosae* development occurs early in development, preceding the third larval instar. In experiment B, development of immature beetles was accelerated under constant dark (0:24) conditions compared to long day photoperiod (17:7) exposure. Interpretation of these data is limited by experimental constraints (two tested photoperiods at a single temperature), however, I do speculate that photoperiod may interact with well-established life stage specific temperature thresholds (Bentz et al. 1991) to influence life cycle coordination across latitudes.

There are examples in the literature of photoperiodic effects on development rate, including diapause programming, for both subcortical tree-tissue developing (Shintani et al. 1996, Doležal and Sehnal 2007, Shintani 2011) and free-living beetles (Doležal et al. 2007, Lopatina et al. 2011) that support my findings for *D. ponderosae*. As briefly reviewed in the introduction (see Dormancy in bark beetles), exposure of immature *I. typographus* to specific photoperiodic conditions induces diapause in the adult stage (Doležal and Sehnal 2007). Doležal & Sehnal (2007) found that diapause was induced when *I. typographus* third instar larvae were reared to pupae under short day and constant temperature conditions, indicating that the immature insects were able to perceive light from beneath the bark and store this photoperiodic information. Diapause was also induced by exposure to short day lengths from the third instar or pupa through to the adult stage, while early instar larvae do not exhibit this sensitivity to diapause inducing cues (at 20°C). This effect could be modified by temperature, as high temperatures characteristic of warm summers were shown to supress the effect of photoperiod on diapause induction. The authors also implicate photoperiod in maintaining diapause in *I. typographus* adults residing within bark or forest litter, as diapause was terminated after transfer to warm temperatures under long day but not short day conditions, and this sensitivity was lost after two months of exposure to cold temperatures (Doležal and Sehnal 2007, Jönsson et al. 2011).

Effects of photoperiod on immature development, diapause induction and number of larval instars are described for the yellow-spotted longicorn beetle (*Psacothea hilaris*) (Shintani et al. 1996) and the chrysanthemum longicorn beetle (*Phytoecia rufiventris*) (Cerambycidae) (Shintani 2011). Like *D. ponderosae* and *I. typographus*, the larvae of both species live and develop within the subcortical tissues of host plants (Shintani et al. 1996, Shintani 2011). Exposure to short day lengths at constant temperature (25°C) lead to the development of one or two supernumerary larval instars and the subsequent induction of diapause in *P. hilaris* (Shintani et al. 1996). Diapause was maintained under short day lengths for more than five months, and terminated by exposure to long day lengths under which pupation occurred. *P. rufiventris* exhibited the opposite pattern, with long day lengths inducing a summer diapause that is terminated by exposure to short

day lengths, ensuring pupation in the early fall and the production of overwintering adults (Shintani 2011). Development up to the third larval instar in *P. rufiventris* was independent of photoperiod, while the development time of older instars differed significantly by treatment; pupation occurred under short day lengths while supernumerary instars and diapause induction resulted from long day exposure. Direct parallels to the effects of photoperiod on *D. ponderosae* development aside, these examples of photoperiodism in immature beetles developing within subcortical plant tissues provide considerable support to my data interpretation.

The Colorado potato beetle (*Leptinotarsa decemlineata,* Chrysomelidae) (Doležal et al. 2007) and *Amara communis* (Carabidae) (Lopatina et al. 2011) are free-living beetles that each exhibit accelerated development under short day photoperiods. Total development rate in *L. decemlineata* is influenced by both temperature and photoperiod, with short day lengths resulting in faster larval development and the induction of adult diapause (de Wilde et al. 1959, Doležal et al. 2007). Larval development and growth rates in the carabid beetle *A. communis* are highly influenced by both photoperiod and temperature (Lopatina et al. 2011). Larvae were found to develop faster and display increased growth rates under short day photoperiods across tested temperatures, facilitating the completion of development and overwintering success in the adult stage. Development rate exhibited a steeper response to temperature under short day photoperiods, an example of photoperiodic modification of thermal reaction norms. Based on my finding of increased development rate in immature *D. ponderosae* under two photoperiods at constant temperature, I propose that thermal reaction norms in the mountain pine beetle may likewise be subject to modification by photoperiod.

Effect of photoperiod on emergence rate

Results from both experiments A and B provide strong support for an effect of photoperiod on *D. ponderosae* emergence rates, which does not appear to depend on rearing photoperiod prior to the third instar. In both cases, adult beetles displayed steeper emergence peaks and somewhat truncated emergence periods under constant darkness (0:24, experiments A ad B) and short day lengths (10:14, experiment A) compared to

insects emerging under long day lengths (17:7, experiments A and B) or constant light (24:0, experiment A). This effect is quite strong, and could contribute to *D. ponderosae* emergence phenology across latitudes. As mountain pine beetle emergence timing is primarily determined by temperature and immature development rate, which is also a function of temperature (Safranyik and Carroll 2006), I propose that a photoperiod-temperature interaction effect on *D. ponderosae* emergence rate may exist across its range.

The relationship between photoperiod and emergence rate is likely driven by seasonal constraints on phenology, that overwintering at any life stage other than mature larvae increases mortality due to cold (Safranyik and Carroll 2006). The exertion of this selective pressure probably guided the evolution of the life stage specific temperature thresholds (Bentz et al. 1991), and may also be implicated in *D. ponderosae* seasonal photoperiodism. Steeper peaks and truncated emergence periods at ~18°C under perceived constant darkness and short day lengths characteristic of late fall could represent a strategy by the beetles to emerge and reproduce at favourable temperatures before the arrival of winter, providing their broods with potential opportunity to reach the fourth instar and successfully overwinter. Beetles exhibiting shallower peaks and extended emergence periods under both long day lengths and constant light conditions may reflect the suboptimal temperature of 18°C for natural summer emergence synchronicity, as emergence generally occurs at temperatures above 20°C in the field (Safranyik and Carroll 2006). Unlike *D. ponderosae*, *I. typographus* overwinters in adult reproductive diapause induced by short day length, while long days encourage swarming (Doležal and Sehnal 2007). Consequently, *I. typographus* exhibit steeper emergence under long day than short day conditions at 20°C, which is a favourable temperature for flight and reproduction (Doležal and Sehnal 2007, Jönsson et al. 2011).

While I have presented experimental data that reveal a photoperiodic emergence response in *D. ponderosae*, previous studies have demonstrated diurnal emergence rhythmicity in mountain pine beetles reared under constant temperature and light conditions, apparently providing evidence of circadian regulation (Watson 1970, Billings and Gara 1975,

Safranyik 1978, Safranyik et al. 1989). Watson (1970) observed a rhythmic daily emergence pattern in mountain pine beetle adults that were reared from the larval stage in field-infested bolts in the dark and under constant temperature. This result suggests that larvae are able to receive light from beneath the bark, and that photoperiodic entrainment of the circadian clock is maintained through to the adult stage. In sum, Watson (1970), Billings and Gara (1975) and Safranyik (1978) provide evidence for endogenous regulation that I speculate may result from circadian entrainment in larvae. I suggest that light input for circadian regulation and photoperiodism (the photoperiodic effects on development and emergence rates uncovered by this study) is likely mediated by the long wavelength opsin that is expressed across *D. ponderosae* life stages, including eggs and eyeless larvae (see CHAPTER 2).

The transmission of both ultraviolet and long wavelength light through existing bark exit holes may provide cues for *D. ponderosae* emergence, as adult beetles also express an ultraviolet opsin the compound eyes (see CHAPTER 2). Reid (1963) observed that mountain pine beetle teneral adults create emergence holes well before exiting the tree, suggesting that the beetles wait within the phloem for optimal temperature (and light) conditions for flight (Shepherd 1966). In addition, Reid (1963) found the average number of adult beetles emerging per exit hole to be 1.14, indicating that more than one beetle may exit from the same hole, particularly at high densities. The production of emergence holes to the outside of the bark could significantly alter the light microenvironment, specifically by facilitating the entry of ultraviolet wavelengths, which are implicated in insect (including weevil) dispersal (Chen et al. 2012, Wang et al. 2013, Stone et al. 2014, Ilić et al. 2016).

Future directions

It is extremely difficult to differentiate between the effects of temperature, photoperiod and dormancy on insect life cycles and seasonal synchrony, primarily due to interactions among factors (Bradshaw and Holzapfel 2007). This statement is particularly true for the mountain pine beetle, which is a challenging experimental organism due to the nature of its within-tree habitat. Seasonal environments vary dramatically with respect to climatic

variables on a daily, seasonal and annual basis. My experiments were conducted under highly controlled laboratory conditions to minimize the confounding effects of variation in temperature, thermoperiod, light intensity and shifting photoperiod. This allowed me to begin to tease apart the effects of photoperiod from those of temperature on *D. ponderosae* immature development and adult emergence.

Interactions between photoperiod and temperature on insect phenology abound in nature, with high temperatures generally amplifying long day effects and low temperatures enhancing short day effects (Bradshaw and Holzapfel 2007). Temperature has the potential to modify photoperiodic reaction norms, while photoperiod is also able to influence thermal reaction norms (Lopatina et al. 2011). While the majority of studies on insect photoperiodism use stable photoperiods due to experimental constraints and the desire to minimize confounding variables, in reality day length shifts slightly every day due to seasonality (de Kort 1990). In the future it would be valuable to investigate the phenology of *D. ponderosae* photoperiodic responses to shifting photoperiods corresponding to seasonality. Immature *D. ponderosae* development and adult emergence in response to photoperiod deserves thorough investigation across a range of temperatures and day lengths to identify potential photoperiod-temperature interactions.

Daytime temperatures are generally higher than night time temperatures, resulting in daily thermoperiodic cycles that approximate seasonal shifts in day length (Beck 1982). Thermoperiod is not as reliable a cue as photoperiod due to climatic temperature variation, but there is evidence that photoperiodic effects can be modified by thermoperiod in insects. For example, larval diapause can be induced by thermoperiod European corn borer *Ostrinia nubilalis* in complete darkness (Beck 1982), while female *Nasonia vitripennis* parasitic wasps, when reared from the egg stage in darkness, are able to differentiate short from long day thermoperiods and produce either diapausing or developing broods in response (Saunders 1973). While it is possible that *D. ponderosae* use thermoperiod as a proxy for photoperiod from beneath the bark (Lester and Irwin 2012), the minimal thermoperiodic variation observed in my experiments does not support an effect of daily temperature cycles on my results. Thermoperiodic treatments

could be incorporated into future experiments examining potential interactions of temperature and photoperiod on *D. ponderosae* phenology.

One interesting follow-up experiment that comes to mind is an investigation into potential photoperiodic entrainment of immature *D. ponderosae* and its downstream circadian emergence rhythmicity. A simple development experiment could be implemented to determine whether photoperiodic entrainment is responsible for the endogenous daily emergence rhythm observed in the mountain pine beetle (Watson 1970, Billings and Gara 1975, Safranyik 1978, Safranyik et al. 1989). If clock entrainment by photoperiod occurs in larval *D. ponderosae*, beetles reared from egg in constant darkness should exhibit no defined emergence rhythm, while insects reared under stable photoperiodic lab conditions should exhibit circadian emergence rhythmicity.

In conclusion, seasonal synchronicity is integral to the life cycle of the mountain pine beetle, and more developmental experiments are required to tease apart the effects of photoperiod on *D. ponderosae* life cycle coordination from those of temperature. My identification of photoperiodic effects on both emergence and immature development rates may eventually lead to improved predictive models for mountain pine beetle outbreaks and range expansion. Further investigations into light sensitivity and maintenance of seasonal rhythms in *D. ponderosae* should enhance our understanding of population dynamics across latitudes, and possibly inform the future management of forest resources.

REFERENCES

Amman, G. D. 1973. Population changes of the mountain pine beetle in relation to elevation. Environmental Entomology 4:541–547.

Beck, S. D. 1982. Thermoperiodic induction of larval diapause in the European corn borer, *Ostrinia nubilalis*. Journal of Insect Physiology 28:273–277.

Belozerov, V. N. 2009. New aspects in investigations of diapause and non-diapause dormancy types in insects and other arthropods. Entomological Review 89:127–136.

Bentz, B. J., J. A. Logan, and G. D. Amman. 1991. Temperature-dependent development of the mountain pine beelte (Coleoptera: Scolytidae) and simulation of its phenology. The Canadian Entomologist 123:1083–1094.

Bentz, B. J., R. R. Bracewell, K. E. Mock, and M. E. Pfrender. 2011. Genetic architecture and phenotypic plasticity of thermally-regulated traits in an eruptive species, *Dendroctonus ponderosae*. Evolutionary Ecology 25:1269–1288.

Bentz, B., J. Vandygriff, C. Jensen, T. Coleman, P. Maloney, S. Smith, A. Grady, and G. Schen-langenheim. 2013. Mountain pine beetle voltinism and life history characteristics across latitudinal and elevational gradients in the western United States. Forest Science 60:434–449.

Billings, R. F., and R. I. Gara. 1975. Rhythmic emergence of *Dendroctonus ponderosae* (Coleoptera: Scolytidae) from two host species. Annals of the Entomological Society of America 68:1033–1036.

Bracewell, R. R., M. E. Pfrender, K. E. Mock, and B. J. Bentz. 2013. Contrasting geographic patterns of genetic differentiation in body size and development time with reproductive isolation in *Dendroctonus ponderosae* (Coleoptera: Curculionidae, Scolytinae). Annals of the Entomological Society of America 106:385–391.

Bradshaw, W. E., and C. M. Holzapfel. 2001. Genetic shift in photoperiodic response correlated with global warming. Proceedings of the National Academy of Sciences of the United States of America 98:14509–14511.

Bradshaw, W. E., and C. M. Holzapfel. 2007. Evolution of Animal Photoperiodism. Annual Review of Ecology, Evolution, and Systematics 38:1–25.

Bradshaw, W. E., and C. M. Holzapfel. 2010. What Season Is It Anyway? Circadian Tracking vs. Photoperiodic Anticipation in Insects. Journal of Biological Rhythms 25:155–165.

Bradshaw, W. E., C. M. Holzapfel, and D. Mathias. 2006. Circadian rhythmicity and photoperiodism in the pitcher-plant mosquito: Can the seasonal timer evolve independently of the circadian clock? American Society of Naturalists 167:601–605.

Bradshaw, W. E., M. C. Quebodeaux, and C. M. Holzapfel. 2003. Circadian rhythmicity and photoperiodism in the pitcher-plant mosquito: adaptive response to the photic environment or correlated response to the seasonal environment? The American Naturalist 161:735–748.

Bünning E. 1936. Die endogene Tagesrhythmik als Grundlage der photoperiodischen Reaktion. Berichte der Deutschen Botanischen Gesellschaft. 54:590-607.

Chen, Y., C. W. Luo, R. P. Kuang, H. W. Li, Z. Chen, and Y. J. Liu. 2012. Phototactic behavior of the Armand pine bark weevil, *Pissodes punctatus*. Journal of Insect Science 13:1–10.

Cornwall C., A. Horiuchi and C. Lehman. 2017. NOAA ESRL Sunrise/Sunset Calculator. Department of Commerce, United States of America: National Oceanic and Atmospheric Administration. URL https://www.esrl.noaa.gov/gmd/grad/solcalc/sunrise.html.

Doležal, P., O. Habuštová, and F. Sehnal. 2007. Effects of photoperiod and temperature on the rate of larval development, food conversion efficiency, and imaginal diapause in *Leptinotarsa decemlineata*. Journal of Insect Physiology 53:849–857.

Doležal, P., and F. Sehnal. 2007. Effects of photoperiod and temperature on the development and diapause of the bark beetle *Ips typographus*. Journal of Applied Entomology 131:165–173.

Emerson, K. J., S. J. Dake, W. E. Bradshaw, and C. M. Holzapfel. 2009. Evolution of photoperiodic time measurement is independent of the circadian clock in the pitcher-plant mosquito, *Wyeomyia smithii*. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology 195:385–391.

Hansen, E. M., B. J. Bentz, J. a. Powell, D. R. Gray, and J. C. Vandygriff. 2011. Prepupal diapause and instar iv developmental rates of the spruce beetle, *Dendroctonus rufipennis* (Coleoptera: Curculionidae, Scolytinae). Journal of Insect Physiology 57:1347–1357.

Hut, R. A., S. Paolucci, R. Dor, C. P. Kyriacou, and S. Daan. 2013. Latitudinal clines: an evolutionary view on biological rhythms. Proceedings of the Royal Society B: Biological Sciences 280:1–9.

Ikeno, T., S. I. Tanaka, H. Numata, and S. G. Goto. 2010. Photoperiodic diapause under the control of circadian clock genes in an insect. BMC Biology 8:116.

Ilić, M., P. Pirih, and G. Belušič. 2016. Four photoreceptor classes in the open rhabdom eye of the red palm weevil, *Rynchophorus ferrugineus* Olivier. Journal of Comparative Physiology A 202:203–213.

Jönsson, A. M., S. Harding, P. Krokene, H. Lange, Å. Lindelöw, B. Økland, H. P. Ravn, and L. M. Schroeder. 2011. Modelling the potential impact of global warming on *Ips typographus* voltinism and reproductive diapause. Climatic Change 109:695–718.

Keeling, C. I., H. Henderson, M. Li, M. Yuen, E. L. Clark, J. D. Fraser, D. P. W. Huber, N. Y. Liao, T. Roderick Docking, I. Birol, S. K. Chan, G. a. Taylor, D. Palmquist, S. J. M. Jones, and J. Bohlmann. 2012. Transcriptome and full-length cDNA resources for the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, a major insect pest of pine forests. Insect Biochemistry and Molecular Biology 42:525–536.

de Kort, C. A. D. 1990. Thirty-five years of diapause research with the Colorado potato beetle. Entomologia Experimentalis et Applicata 56:1–13.

Koštál, V. 2006. Eco-physiological phases of insect diapause. Journal of Insect Physiology 52:113–127.

Lester, J. D., and J. T. Irwin. 2012. Metabolism and cold tolerance of overwintering adult mountain pine beetles (*Dendroctonus ponderosae*): Evidence of facultative diapause? Journal of Insect Physiology 58:808–815.

Lopatina, E. B., V. E. Kipyatkov, S. V. Balashov, and D. A. Kutcherov. 2011. Photoperiod-temperature interaction-a new form of seasonal control of growth and development in insects and in particular a carabid beetle, *Amara communis* (Coleoptera: Carabidae). Journal of Evolutionary Biochemistry and Physiology 47:578–592.

R Core Team. 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Reid, R. 1963. Biology of the mountain pine beetle, *Dendroctonus monticolae* Hopkins, in the East Kootenay Region of British Columbia. III. Interaction between the beetle and its host, with an emphasis on brood morality and survival. The Canadian Entomologist 95:225–238.

Reid, R. W., and H. Gates. 1970. Effect of temperature and resin on hatch of eggs of the mountain pine beetle (*Dendroctonus ponderosae*). The Canadian Entomologist 102:617–622.

RStudio Team. 2012. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/.

Safranyik, L. 1978. Effects of climate and weather on mountain pine beetle populations. Pages 77–84. Theory and practice of mountain pine beetle mamagement in lodgepole pine forests. Moscow, Idaho.

Safranyik, L., and A. Carroll. 2006. The biology and epidemiology of the mountain pine beetle in lodgepole pine forests. Pages 3-66 (Chapter 1) in L. Safranyik and W.R. Wilson, editors. The mountain pine beetle: a synthesis of biology, management, and impacts on lodgepole pine. Natural Resources Canada, Canadian Forest Service, Pacific Forestry Centre, Victoria, British Columbia.

Safranyik, L., A. L. Carroll, J. Régnière, D. W. Langor, W. G. Riel, T. L. Shore, B. Peter, B. J. Cooke, V. G. Nealis, and S. W. Taylor. 2010. Potential for range expansion of mountain pine beetle into the boreal forest of North America. The Canadian Entomologist 142:415–442.

Safranyik, L., R. Silversides, L. McMullen, and D. Linton. 1989. An empirical approach to modeling the local dispersal of the mountain pine beetle (*Dendroctonus ponderosae* Hopk.) (Col., Scolytidae) in relation to sources of attraction, wind direction and speed. Journal of Applied Entomology 108:498–511.

Safranyik, L., and H. S. Whitney. 1985. Development and survival of axenically reared mountain pine beetles, *Dendroctonus ponderosae* (Coleoptera: Scolytidae), at constant temperatures. The Canadian Entomologist 117:185–192.

Sandrelli, F., E. Tauber, M. Pegoraro, G. Mazzotta, P. Cisotto, J. Landskron, R. Stanewsky, A. Piccin, E. Rosato, M. Zordan, R. Costa, and C. P. Kyriacou. 2007. A molecular basis for natural selection at the timeless locus in *Drosophila melanogaster*. Science 316:1898–1900.

Saunders, D. S. 1973. Thermoperiodic Control of Diapause in an Insect: Theory of Internal Coincidence. American Association for the Advancement of Science 181:358–360.

Saunders, D. S. 2012. Insect photoperiodism: Seeing the light. Physiological Entomology 37:207–218.

Schroeder, M., and P. Dalin. 2016. Differences in photoperiod-induced diapause plasticity among different populations of the bark beetle *Ips typographus* and its predator *Thanasimus formicarius*. Agricultural and Forest Entomology 18:1–8.

Shepherd, R. F. 1966. Factors influencing the orientation and rates of activity of *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae). The Canadian Entomologist 98:507–518.

Shintani, Y. 2011. Quantitative short-day photoperiodic response in larval development and its adaptive significance in an adult-overwintering cerambycid beetle, *Phytoecia rufiventris*. Journal of Insect Physiology 57:1053–1059.

Shintani, Y., Y. Ishikawa, and S. Tatsuki. 1996. Larval diapause in the yellow-spotted longicorn beetle, *Psacothea hilaris* (Pascoe) (Coleoptera: Cerambycidae). Applied Entomology and Zoology 31:489–494.

Stone, T., M. Mangan, P. Ardin, and B. Webb. 2014. Sky segmentation with ultraviolet images can be used for navigation. Proceedings Robotics: Science and Systems: 1–10.

Tauber, E., M. Zordan, F. Sandrelli, M. Pegoraro, N. Osterwalder, C. Breda, A. Daga, A. Selmin, K. Monger, C. Benna, E. Rosato, C. P. Kyriacou, and R. Costa. 2007. Natural selection favors a newly derived timeless allele in *Drosophila melanogaster*. Science 316:1895–1898.

Wang, B., J. H. Xiao, S. N. Bian, L. M. Niu, R. W. Murphy, and D. W. Huang. 2013. Evolution and expression plasticity of opsin genes in a fig pollinator, *Ceratosolen solmsi*. PLoS ONE 8:1–11.

Watson, J. A. 1970. Rhythmic emergence patterns of the mountain pine beetle *Dendroctonus ponderosae* (Coleoptera: Scolytidae). The Canadian Entomologist 102:1054–1056.

Wickham, H. 2009. ggplot2: Elegant graphics for data analysis. Springer-Verlag New York.

de Wilde, J., S .C. Duintjer, and L. Mook. 1959. Physiology of diapause in the adult Colorado beetle (Leptinotarsa decemlineata Say) – I. The photoperiod as a controlling factor. Journal of Insect Physiology 3:75–85.

CHAPTER 3 Tables

Table 1. Results from the generalized linear models used to evaluate the effects of photoperiod on *D. ponderosae* development and emergence rates from experiment A (reared from field-collected third or fourth instar under photoperiod treatments in the laboratory). DD = degree day, Light = photoperiod treatment (0:24, 10:14, 17:7, 24:0), n = number of observations [number of samples used to calculate proportion (binomial), number emerged beetle degree day observations (Poisson)]. Significance of independent variables is denoted by an asterisk ($p < 0.05$).

Model type	Dependent variable	n	Residual df	Independent variables	z-value	p-value
Binomial logistic	Proportion larvae	24	21	DD*	-19.227	< 0.0001
				Light	0.267	0.789
Poisson	DD to emergence	973	971	Light*	-6.682	< 0.0001

Table 2. Results from the generalized linear models used to evaluate the effects of photoperiod on *D. ponderosae* development and emergence rates from experiment B (reared from eggs under photoperiod treatments in the laboratory). DD = degree day, Light = photoperiod treatment (0:24, 17:7), n = number of observations [number of samples used to calculate proportion (binomial), number of pupa/emerged beetle degree day observations (Poisson)]. Significance of independent variables is denoted by an asterisk ($p < 0.05$).

Model type	Dependent variable	n	Residual df	Independent variables	z-value	p-value
Binomial logistic	Proportion larvae	10	7	DD*	-6.605	< 0.0001
				Light*	2.637	< 0.01
Poisson	DD to pupation	69	67	Light*	16.85	< 0.0001
Poisson	DD to emergence	310	308	Light*	20.08	< 0.0001

CHAPTER 3 Figures

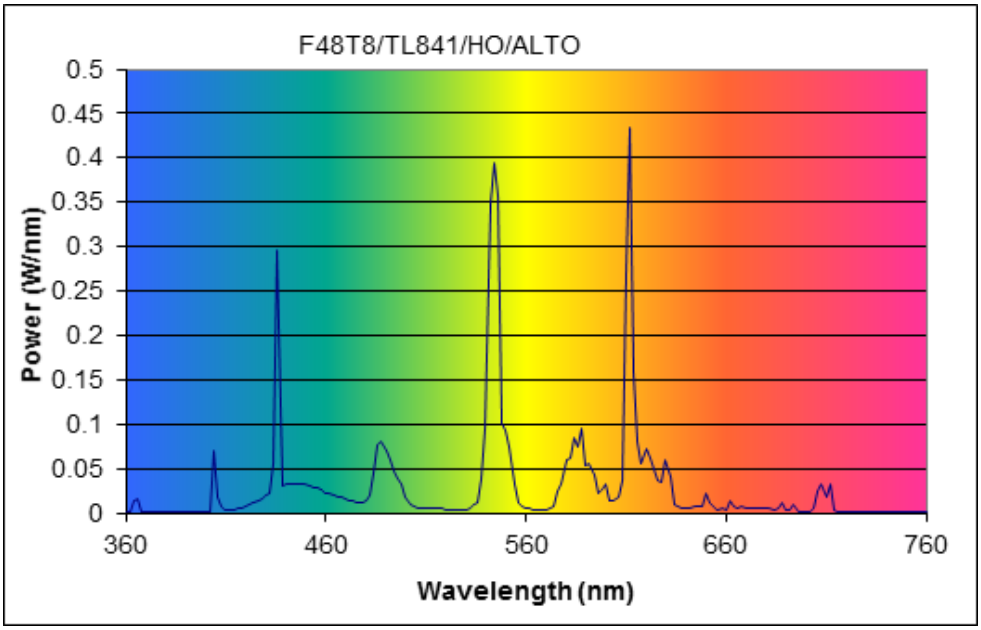


Figure 1. Wavelength output in W/nm for the F48T8/TL841/HO 4100K 44 Watt Hg 2L bulbs used to provide light treatment in development experiments A and B (Philips Lighting Holding B.V. 2017). The bulbs emit broad-spectrum light including wavelengths in the ultraviolet (UVA) range (320-400nm).

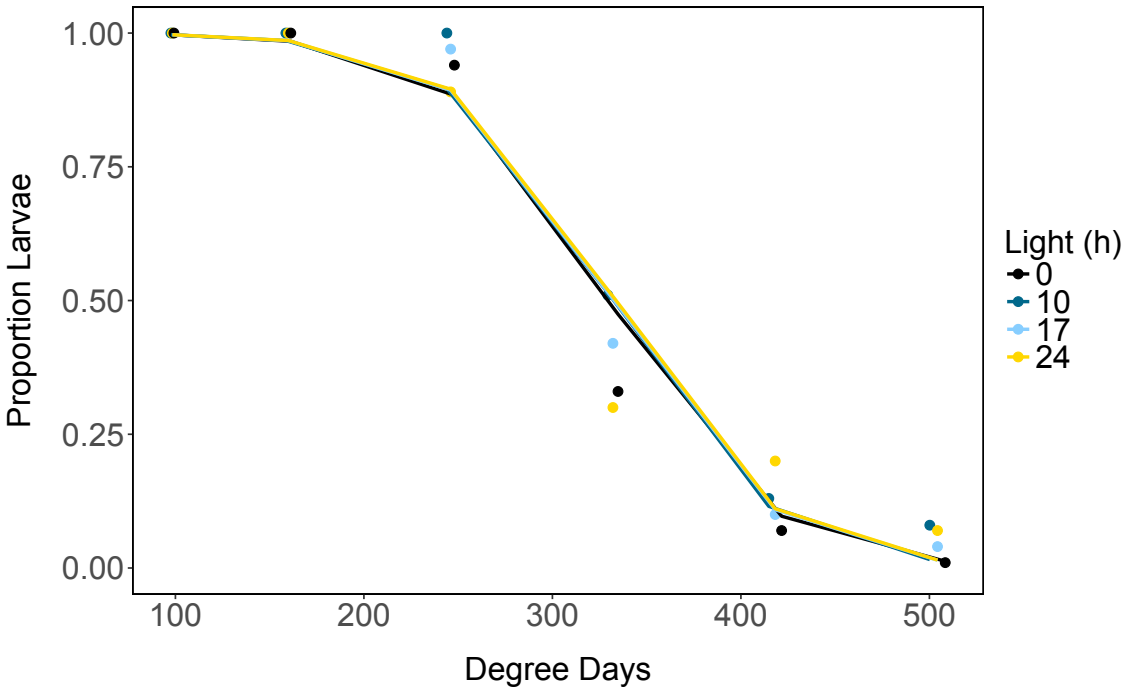


Figure 2. Plot of proportion larvae observed in response to degree day, separated by photoperiod, for experiment A (n = 24 observations across six time points). The fitted binomial model does not support an effect of photoperiod on *D. ponderosae* development rate (z_{21} = 0.267: p = 0.79). Dots represent proportion larvae at each sample time, while lines show the predicted model fit by photoperiod.

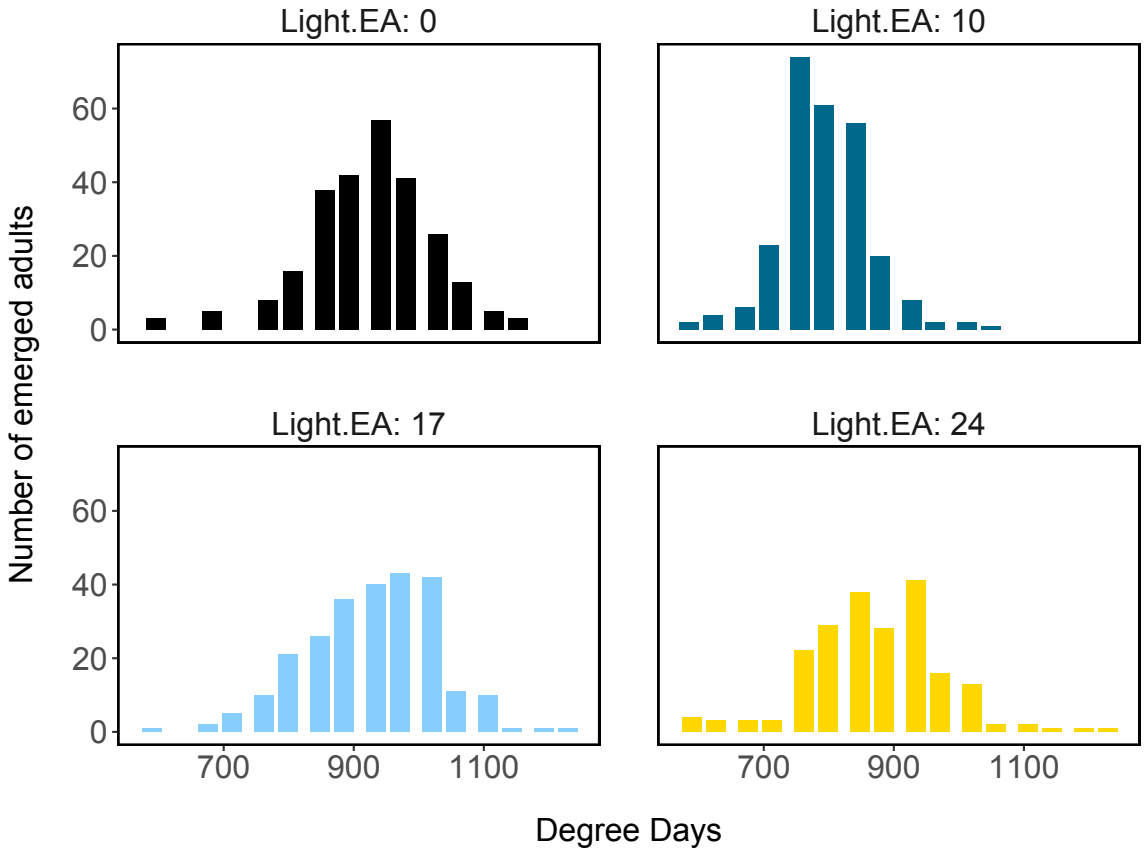


Figure 3. Bar plot of emerged *D. ponderosae* adult counts by degree day, separated by photoperiod, for experiment A (overall n = 973 beetles; 0:24 n = 257, 10:14 n = 259, 17:7 n = 250, 24:0 n = 207). Trends indicate steeper emergence peaks at 0:24 and 10:14 (short day) photoperiods than at 17:7 (long day) and 24:0 photoperiods. There is a significant effect of photoperiod on number of degree days to emergence (Poisson model: $z_{971} = -6.682$: $p < 0.0001$).

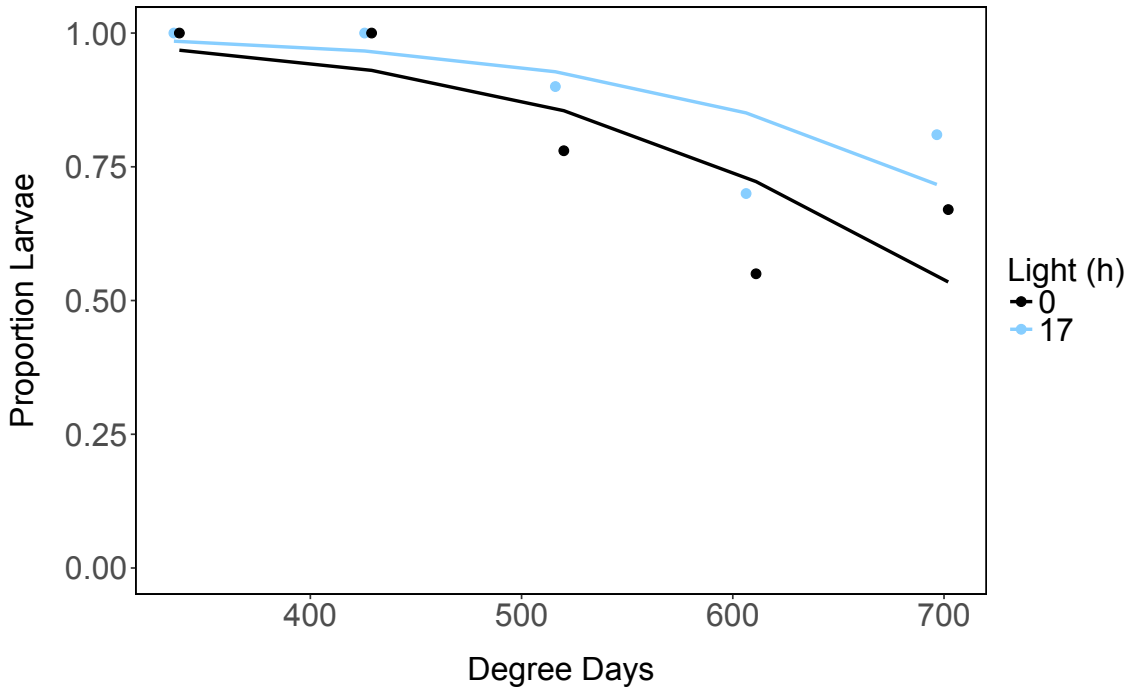


Figure 4. Plot of proportion larvae in response to degree day, separated by photoperiod, for experiment B sample (n = 10 observations across five time points). Analysis reveals a significant effect of photoperiod on immature *D. ponderosae* development rate (binomial model: $z_7 = 2.637$: $p < 0.01$). Dots represent proportion larvae at each sample time, while lines show the predicted binomial model fit by photoperiod (0:24 or 17:7).

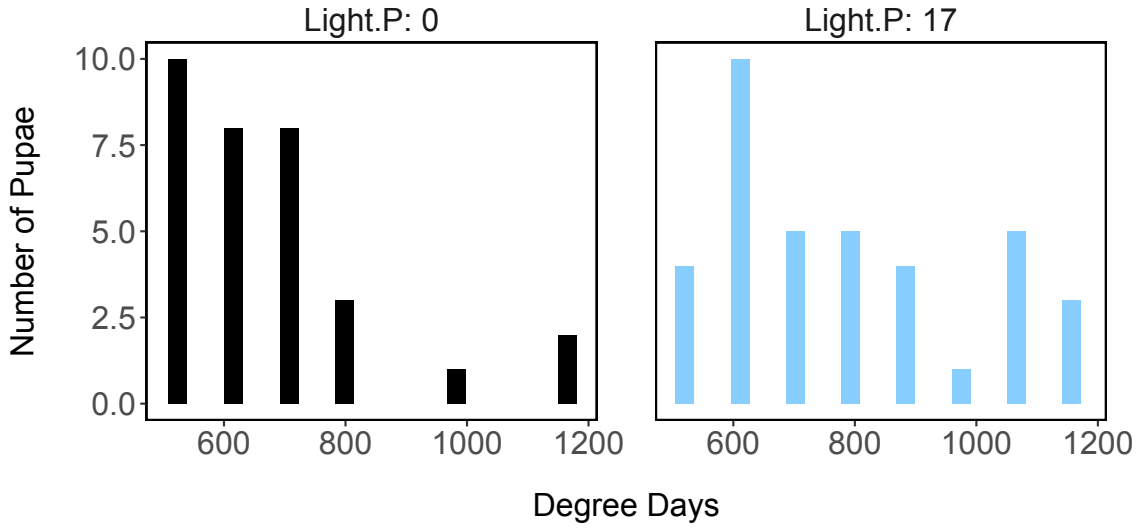


Figure 5. Bar plot displaying number of *D. ponderosae* pupae in response to degree day, separated by photoperiod, for experiment B (overall n = 69 pupae, 0:24 n = 32, 17:7 n = 37). Beetles appear to pupate earlier under constant darkness than at 17:7. There is a significant effect of photoperiod on degree days to pupation (Poisson model: $z_{67} = 16.85$: $p < 0.0001$).

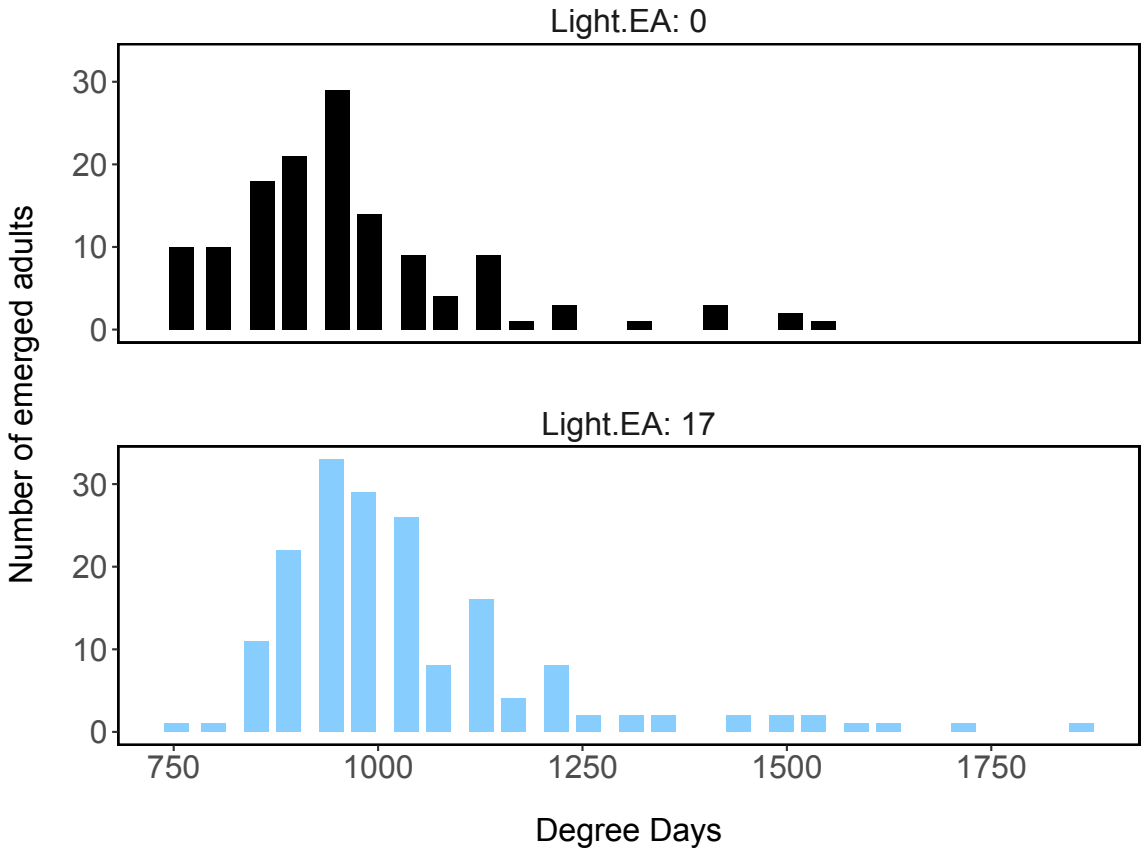


Figure 6. Bar plot of the number of emerged *D. ponderosae* adults in response to degree day, separated by photoperiod, from experiment B (overall n = 310 beetles; 0:24 n = 114, 17:7 n = 175). The beetles exhibit a steeper emergence peak in constant darkness than under long day (17:7) conditions. Analysis reveals a significant effect of photoperiod on number of degree days to emergence (Poisson model: z_{308} =20.08: $p < 0.0001$).

CHAPTER 3 Appendix

Appendix Table 1. Output of the experiment A binomial logistic model used to test for an effect of photoperiod (Light.h; 0:24, 10:14, 17:7, 24:0) and degree day (DD) on proportion *D. ponderosae* larvae sampled over time (significance level *** = p < 0.001) (RStudio Team 2015, R Core Team 2015).

	Estimate	**Std. Error**	**z-value**	**Pr(>|z|)**
(Intercept)	8.116530	0.448900	18.081	<2e-16 ***
DD	-0.024549	0.001277	-19.227	<2e-16 ***
Light.h	0.002794	0.010457	0.267	0.789

Null deviance: 1483.289 on 23 degrees of freedom
Residual deviance: 76.707 on 21 degrees of freedom

Appendix Table 2. Output of the experiment A Poisson model used to test for an effect of photoperiod (Light.EA; 0:24, 10:14, 17:7, 24:0) on degree days to adult emergence (significance level *** = p < 0.001) in *D. ponderosae* (RStudio Team 2015, R Core Team 2015).

	Estimate	**Std. Error**	**z-value**	**Pr(>|z|)**
(Intercept)	6.7895332	0.0018447	3680.640	< 2e-16 ***
Light.EA	-0.0008273	0.0001238	-6.682	2.36e-11 ***

Null deviance: 12898 on 972 degrees of freedom
Residual deviance: 12853 on 971 degrees of freedom

Appendix Table 3. Output of the experiment B binomial logistic model used to test for an effect of photoperiod (Light.h; 0:24, 17:7) and degree day (DD) on proportion *D. ponderosae* larvae sampled over time (significance level *** = p < 0.001, ** = p < 0.01) (RStudio Team 2015, R Core Team 2015).

	Estimate	**Std. Error**	**z-value**	**Pr(>|z|)**
(Intercept)	6.44283	0.83293	7.735	1.03e-14 ***
DD	-0.00898	0.00136	-6.605	3.97e-11 ***
Light.h	0.74202	0.28140	2.637	0.00837 **

Null deviance: 98.222 on 9 degrees of freedom
Residual deviance: 34.891 on 7 degrees of freedom

Appendix Table 4. Output of the experiment A Poisson model used to test for an effect of photoperiod (Light.P; 0:24, 17:7) on degree days to pupation (significance level *** = p < 0.001) in *D. ponderosae* (RStudio Team 2015, R Core Team 2015).

	Estimate	**Std. Error**	**z-value**	**Pr(>|z|)**
(Intercept)	6.511764	0.006814	955.62	<2e-16 ***
Light.P	0.151576	0.008997	16.85	<2e-16 ***

Null deviance: 3352.9 on 68 degrees of freedom
Residual deviance: 3067.2 on 67 degrees of freedom

Appendix Table 5. Output of the experiment B Poisson model used to test for an effect of photoperiod (Light.EA; 0:24, 17:7) on degree days to adult emergence (significance level *** = p < 0.001) in *D. ponderosae* (RStudio Team 2015, R Core Team 2015).

	Estimate	**Std. Error**	**z-value**	**Pr(>|z|)**
(Intercept)	6.871995	0.002771	2480.19	<2e-16 ***
Light.EA	0.072919	0.003631	20.08	<2e-16 ***

Null deviance: 8258.8 on 309 degrees of freedom
Residual deviance: 7853.9 on 308 degrees of freedom

See attached files:

Chapter3Appendix_ExperimentA.xlsx
Appendix Table 6. Experiment A sample data.
Appendix Table 7. Experiment A emergence data.

Chapter3Appendix_ExperimentB.xlsx
Appendix Table 8. Experiment B sample and emergence data.