**Sociality in Caterpillars: Investigations into the Mechanisms
Associated with Grouping Behaviour, from Vibroacoustics to
Sociogenomics**

by

Chanchal Yadav

A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in
partial fulfillment of the requirements for the degree of

Doctorate of Philosophy
in
Biology

Carleton University
Ottawa, Ontario

© 2020, Chanchal Yadav

Abstract

Social grouping is widespread among larval insects, particularly in a number of phytophagous larval Lepidoptera (caterpillars). Although the benefits of social grouping are widely recognized, the proximate mechanisms mediating grouping behaviour, such as group formation and maintenance, are poorly understood. My Ph.D. thesis takes a pioneering approach to understanding these mechanisms, specifically, by studying the roles of vibroacoustics and sociogenomics, using the masked birch caterpillar, *Drepana arcuata* (Lepidoptera: Drepanoidea), as a model. There are two main objectives of my thesis - (i) to test the hypothesis that caterpillars employ plant-borne vibratory signals to recruit conspecifics to social groups; and (ii) to test the hypothesis that differential gene expression is associated with developmental transitions from social to solitary behavioural states. For the first objective, I documented morphological and behavioural changes in the larvae, showing that there are five larval instars, and developmental changes in social and signalling behaviour. Specifically, early instars (I, II) live in small social groups, and late instars (IV, V) live solitarily, with third instars (III) being transitional. Instars I-III generate four signal types (AS, BS, MS, MD), instars IV, V generate three signals (AS, MS, MD). I then used an experimental approach to test if early instars employ vibrations during social recruitment, and found that vibratory signals are used to advertise feeding and silk shelters, leading to recruitment, with higher signalling rates resulting in faster joining times by conspecifics. For the second objective, comparative transcriptomic analysis indicates that there are 3300 transcripts differentially expressed between early (social) and late (solitary) instars, and these include transcripts potentially coding for candidate ‘social’ genes. One of these genes- an octopamine receptor gene- was further functionally tested using RNAi,

and preliminary results suggest that its reduced expression is associated with hastened social to solitary transition. As this research contributes the first genomic data on an entire lepidopteran superfamily (Drepanoidea), I also assembled a draft genome of *D. arcuata*. The research is the first to test hypotheses on the roles of vibrational signalling and genomics in the social behaviour of larval insects, many of which are of great economic and ecological importance.

Acknowledgements

I would like to begin with thanking my Ph.D. supervisor, Dr. Jayne Yack. Words fall short as I begin writing how grateful I am to have had Dr. Yack as my supervisor. Right from the day I walked into her office with an international undergrad degree (and all the complications that go into applying to grad school with that), she has been wonderful mentor, providing tremendous support in everything that I pursued, both as a researcher and as an individual. Not only did she put phenomenal work in to helping me with my research, but also, she always encouraged me and provided me with numerous opportunities and support to present my work at different platforms. Thank you for always providing the opportunity to work with you, for believing in me, making me realize my true potential, for always pushing me to succeed, to strive for excellence, for all the cheers, for picking me up every time I was down, the list is endless! Having her as a supervisor made grad school such a wonderful experience for me that I will cherish forever. She has played the biggest role in my ‘metamorphosis’, both as a researcher as well as a person. I will always remember her words “hard work + perseverance= success”. With all the personal and professional advices that I have received from you over the years, I can safely assume you’re like my second mother who always has my best interests at heart.

A big thanks goes to Dr. Myron Smith (committee), for his invaluable support, constant guidance and encouragement throughout my doctorate. Thank you for helping me with all the molecular work in lab, in finding things in your lab, for the honey that you ‘stole’ from bees, and for always coming up with new experimental ideas to try with drepanids, which reminds me we should really finish those pesticide trials. Thanks to Dr. Alex Wong (committee) for introducing me to bioinformatics, and for the invaluable

feedback on my research through these years. Thank you to Dr. Rex Cocroft, Dr. Jessica Forrest and Dr. Alfonso Abizaid for kindly agreeing to participate as examiners in my Ph.D. defense.

Thanks to everyone from Yack lab (both past and present members) that I have met over the years for their support, feedbacks and friendships. Special thanks to Amanda D., Amanda L., Andras, Conrado, Melanie, Mairelys, Elham, and Leo for being my friends, motivators and the best labmates I could have ever asked for. Also, thanks to members of Smith lab –Ghazaleh, Bodunde, Shravan, Tom, Emma, and Anatoly for their constant support and advice in the lab. Thanks to my fellow grads, especially Kamya and Ghazaleh for their friendships, and amazing memories. A big thanks to the amazing faculty and staff at Carleton University Biology Department, especially Darlene Moss, SarahAnne Szaboth, Caitlyn McKenzie, Jennifer Harris, and the late Laura Thomas, for always helping me and answering questions. Thanks to everyone who I have met with or been a co-TA with at Carleton, as well as at conferences and workshops over the years, I have always learnt so much from each and every one of you, leading to my professional and personal growth.

A big thanks to everyone who helped with data collection and analysis for my project. Thank you Upendra DeviSetty for your unwavering support with bioinformatics analyses, right from resource allocation to data analysis. I am so grateful to have met you at the ANGUS workshop. Thanks to Mairelys Naranjo for help with acoustic analyses; Raul Guedes and Leo Turchen for statistical analyses; to Jake Miall for help with insect collection; to Compute Canada, XSEDE and CyVerse for providing me with computational resources and services for the genomics work of my project.

Finally, I would like to thank my parents for letting me to move to this wonderful country that I call home now, it has changed my life only for the better, something that I would always be grateful for. As a final thought I would like to thank the stars of this show- "*Drepana arcuata*" for the opportunity to conduct this research.

This research was funded by the Natural Science and Engineering Research Council of Canada (NSERC) Discovery Grant, and the Canadian Foundation for Innovation (CFI) to Dr. Jayne Yack. Additional funding sources that aided in the completion of my doctoral program include Carleton Biology departmental scholarships (Bonneville Family Scholarship in Botany, Hibiscus Millennium Project Bursary), and the Ontario Graduate Scholarships. I am grateful to all the funding sources as they not only provided me with the opportunity to complete my doctoral program, but also expanded the horizons of my scientific knowledge by enabling me travel to the UC Davis for bioinformatics workshop and to attend several national and international conferences.

Table of Contents

Abstract.....	ii
Acknowledgements	iv
Table of Contents	vii
List of Tables	xii
List of Appendices.....	xvi
Chapter 1: General Introduction	1
1.1 General overview.....	2
1.2 Social behaviour defined	3
1.3 Ultimate and proximate mechanisms of social behaviour	4
1.4 Vibroacoustics and the role of vibrations in grouping behaviour.....	5
1.5 Sociogenomics and the genomic influences on grouping behaviour.....	7
1.6 Study organism: The masked birch caterpillar, <i>Drepana arcuata</i>	8
1.7 Major thesis goals.....	8
Chapter 2: Immature stages of the masked birch caterpillar, <i>Drepana arcuata</i> (Lepidoptera: Drepanidae) with comments on feeding and shelter building.....	10
2.1 Abstract	11
2.2 Introduction	12
2.3 Methods.....	15
2.3.1 <i>Insect collection and rearing</i>	15
2.3.2 <i>Morphology</i>	16
2.3.3 <i>Behavioural observations</i>	17
2.3.4 <i>Measurements to distinguish larval stages</i>	17
2.4 Results	18
2.4.1 <i>General comments on immature stages and rearing</i>	18

2.4.2	<i>Eggs</i>	18
2.4.3	<i>First instar</i>	21
2.4.4	<i>Second instar</i>	26
2.4.5	<i>Third instar</i>	27
2.4.6	<i>Fourth instar</i>	28
2.4.7	<i>Fifth instar</i>	29
2.4.8	<i>Pupa</i>	30
2.4.9	<i>Comparisons between sizes of instars</i>	32
2.5	Discussion	34
 Chapter 3: Developmental changes in social grouping and vibratory signalling in the masked birch caterpillar, <i>Drepana arcuata</i> (Drepanidae)		38
3.1	Abstract	39
3.2	Introduction	40
3.3	Methods	44
3.3.1	<i>Study animals</i>	44
3.3.2	<i>Group formation trials</i>	44
3.3.3	<i>Types of vibratory signals and variation within and between instars</i>	48
3.4	Results	53
3.4.1	<i>Group formation throughout development</i>	53
3.4.2	<i>Variation in signal types and rates between larval instars</i>	59
3.5	Discussion	77
 Chapter 4: Invitation by vibration: recruitment to feeding shelters by vibrating caterpillars		81
4.1	Abstract	82
4.2	Significance statement	83
4.3	Introduction	83

4.4	Methods	87
4.4.1	<i>Study animals</i>	87
4.4.2	<i>Group formation experiment</i>	88
4.4.3	<i>Y-choice experiments</i>	91
4.4.4	<i>Vibration signal types and rates</i>	94
4.4.5	<i>Vibratory signalling during group formation</i>	95
4.5	Results	98
4.5.1	<i>Group formation and locations</i>	98
4.5.2	<i>Y choice experiments</i>	101
4.5.3	<i>Vibration signal types</i>	101
4.5.4	<i>Signalling rates and associated behavioural states of residents</i>	101
4.5.5	<i>Vibrational signalling associated with group formation</i>	109
4.6	Discussion.....	113
4.7	Conclusions	118

Chapter 5: Transcriptome analysis of a social caterpillar, *Drepana arcuata*: de novo assembly, functional annotation and developmental analysis 120

5.1	Abstract	121
5.2	Introduction	122
5.3	Material and methods	126
5.3.1	<i>Sample preparation</i>	126
5.3.2	<i>RNA extraction and sequencing</i>	129
5.3.3	<i>De novo transcriptome assembly and annotation</i>	129
5.3.4	<i>Differential transcript expression analysis</i>	130
5.3.5	<i>RT-qPCR validation of differentially expressed transcripts</i>	130
5.4	Results and discussion.....	131
5.4.1	<i>De novo transcriptome assembly and annotation</i>	131

5.4.2	<i>Differentially expressed transcripts in early vs late instars</i>	138
5.5	Conclusion.....	146
Chapter 6: Studying the effects of a candidate “social” gene (octopamine receptor) on the grouping behaviour of early instar <i>D. arcuata</i> using RNAi: a preliminary investigation.....		148
6.1	Abstract	149
6.2	Introduction	150
6.3	Materials and Methods	154
6.3.1	<i>Insect collection and rearing</i>	154
6.3.2	<i>dsRNA design and synthesis</i>	155
6.3.3	<i>dsRNA feeding and behavioural trials</i>	158
6.3.4	<i>Primer design and RT-qPCR</i>	160
6.4	Results and discussion.....	163
6.4.1	<i>Alterations in octopamine receptor transcript expression</i>	163
6.4.2	<i>Group formation trials</i>	166
Chapter 7: Draft genome assembly and annotation of masked birch caterpillar, <i>Drepana arcuata</i> (Lepidoptera: Drepanoidea)		173
7.1	Abstract	174
7.2	Introduction	175
7.3	Experimental Design, Materials and Methods	179
7.3.1	<i>Sample collection and sequencing</i>	179
7.3.2	<i>Genome assembly and annotation</i>	180
7.4	Value of the Data.....	181
Chapter 8: Summary and Conclusions.....		182
8.1.1	Thesis goals	183
8.1.2	Summary of research chapters	184

8.1.3	Final thoughts and future directions	189
Appendices.....		193
	Appendix A Supplementary material for Chapter Four	193
	Appendix B Supplementary material for Chapter Five	195
	Appendix C Supplementary material for Chapter Six	197
	Appendix D Supplementary material for Chapter Seven	201
References		202

List of Tables

Table 2.1 Head capsule measurements for instars (I–V) of <i>D. arcuata</i> .	25
Table 2.2 Body length measurements for instars of <i>D. arcuata</i> .	25
Table 3.1 Characteristics of the vibratory signals produced by third instar <i>D. arcuata</i> .	61
Table 3.2 Signal rates generated by <i>D. arcuata</i> larvae across instars.	65
Table 3.3 Signal rates for each signal type generated by <i>D. arcuata</i> instars I–V.	65
Table 3.4 Signal rates for solitary individuals of <i>D. arcuata</i> instars I–V.	69
Table 3.5 Signal rates for each signal type generated by solitary individuals from <i>D. arcuata</i> instars I–V.	72
Table 4.1 Vibratory signals in early-instar <i>D. arcuata</i> caterpillars during different behavioural states.	105
Table 4.2 (CVA) of the signal rates of early-instar <i>D. arcuata</i> associated with four behavioural states.	108
Table 5.1 Summary of RNA sequencing data and combined early and late instar larval transcriptome.	134
Table 5.2 Some of the most up-regulated transcripts and other interesting DETs in early and late instars.	141
Table 5.3 Examples of social genes/molecular products in invertebrates and their expression in larval <i>D. arcuata</i> transcriptome.	144
Table 6.1 Commercially synthesized siRNAs for <i>D. arcuata</i> OAR transcript sequence	156
Table 6.2 Sequences of OAR and <i>rps7</i> primers used for RT-qPCR.	161
Table 6.3 RT-qPCR and behavioural assay results for individual trials for each dsRNA.	168
Table 7.1 Characteristics of draft genome assembly, gene predictions and functional annotation of <i>D. arcuata</i> .	177
Table 7.2 Summary of <i>D. arcuata</i> draft genome quality assessment done using BUSCO against Arthropoda orthologs.	178

List of Figures

Figure 2.1 Adult moth <i>D. arcuata</i> in resting position on a birch leaf.....	14
Figure 2.2 Eggs of <i>D. arcuata</i> laid in rows on birch leaves.	20
Figure 2.3 Light micrographs and SEMs (right) of <i>D. arcuata</i> head capsules.....	23
Figure 2.4 Lateral views of each instar and dorsal views of larvae within their characteristic shelter.....	24
Figure 2.5 Light micrographs of prepupa and pupa of <i>D. arcuata</i>	31
Figure 2.6 Distribution of head capsule widths and body lengths for <i>D. arcuata</i> instars	33
Figure 2.7 Natural log of mean head capsule widths plotted against the number of instars	33
Figure 3.1 Early and late instar <i>D. arcuata</i> larvae.....	43
Figure 3.2 Set-up used for group formation trials conducted for <i>D. arcuata</i> instars I-V.	47
Figure 3.3 Group formation by <i>D. arcuata</i> instars over a 24 h observation period.....	54
Figure 3.4 Group formation trials for <i>D. arcuata</i> instars I-V..	55
Figure 3.5 Median time taken by <i>D. arcuata</i> instars I-V to form groups..	56
Figure 3.6 Variation in group sizes formed by <i>D. arcuata</i> instars I-V	58
Figure 3.7 Types of signals generated by <i>D. arcuata</i> larvae..	62
Figure 3.8 Scanning electron micrographs (SEMs) of anal oars of <i>D. arcuata</i> larvae....	63
Figure 3.9 Mean signal rates types and group size..	66
Figure 3.10 Mean signal rates for each signal type generated by <i>D. arcuata</i> instars I-V.	67
Figure 3.11 Mean signal rates per individual generated by solitary <i>D. arcuata</i> larvae across instars I-V.....	70
Figure 3.12 Signal rates for each signal type generated by solitary individuals from <i>D. arcuata</i> instars I-V..	73
Figure 3.13 Signal rates for individuals across group sizes of 1-5	75

Figure 3.14 Signal rates for each signal type for individuals across group sizes of 1-5..	76
Figure 4.1 Eggs and early-instar larvae of <i>D. arcuata</i>	86
Figure 4.2 Group formation experiment..	90
Figure 4.3 Y-choice experiments testing preferences of test larvae for joining conspecific.	93
Figure 4.4 Vibration signals.....	100
Figure 4.5 Vibration signal types and rates for solitary early-instar <i>D. arcuata</i> larvae in shelters..	103
Figure 4.6 Relationships between behavioural states and vibratory signals in early-instar <i>D. arcuata</i> larvae..	106
Figure 4.7 Vibratory signalling during grouping events in early-instar <i>D. arcuata</i>	110
Figure 4.8 Vibration signal rates relative to the duration and success of a recruitment event.	112
Figure 5.1 Selected developmental stages of <i>D. arcuata</i>	125
Figure 5.2 Summary of methods used for transcriptome assembly and differential transcript expression analysis..	128
Figure 5.3 Homology of transcripts to Non-Redundant (NR) protein database.	136
Figure 5.4 Gene Ontology (GO) classification of larval transcriptome.....	137
Figure 5.5 Heat map showing transcripts that are differentially expressed between early and late instars.....	140
Figure 6.1 Diagram representing the octopamine receptor (OAR) transcript, the locations of three dsRNAs used for RNAi experiments and primers used for RT-qPCR.....	157
Figure 6.2 An overview of methods employed in the current study testing the influence of an OAR gene using RNAi on the social behavioural transition in <i>D. arcuata</i> caterpillars.....	159
Figure 6.3 OAR transcript abundance after 48 h of dsOAR-2 and dsOAR-3 treatments or DEPC water controls).	165

Figure 6.4 Bar graph representing the percentage of larvae that remained solitary at the end of behavioural trials..... 167

Figure 7.1 Summary of methods used for draft genome assembly and annotation of *Drepana arcuata*. 176

List of Appendices

Appendix A Supplementary material for Chapter Four	193
Figure A4.1 Waveform of the laser vibrometer recording.....	193
File A4.1 Laser vibrometer recording (.wav file) with 4 signal types generated by an early instar <i>D. arcuata</i>	194
File A4.2 m4v file showing AS generated by an early instar <i>D. arcuata</i> during feeding and silk shelter construction.....	194
File A4.3 m4v file showing different vibratory signals generated during recruitment event, both by resident as well as the joining conspecific early instars <i>D. arcuata</i>	194
Appendix B Supplementary material for Chapter Five	195
Figure B5.1 Length distribution of the larval <i>D. arcuata</i> transcripts assembled by Trinity.	195
Figure B5.2 Representation of partitioning of <i>D. arcuata</i> transcripts into KEGG pathways.	195
Figure B5.3 Histogram representing classification of clusters of orthologous groups (KOG) for the larval transcriptome.....	195
Figure B5.4 Biological validation of RNA-seq-based DETs using RT-qPCR).....	195
Figure B5.5 Representation of partitioning of DETs into KEGG pathways..	195
Figure B5.6 Gene Ontology (GO) classification of differentially expressed transcripts between early and late instar <i>D. arcuata</i>	196
Table B5.1 Availability of data used in the transcriptomic analysis of larval <i>D. arcuata</i>	196
Table B5.2 Transcript IDs and sequences of primers used for RT-qPCR validation of selected DETs in late vs early instars.	196
Table B5.3 Summary of RNA sequencing data and transcriptome assembly for early and late instar larvae.	196
File B5.1 Trinotate annotation report for larval <i>D. arcuata</i> transcriptome.	196

File B5.2 Differential expression information on DETs ($\log_2FC \geq 2$; $FDR \leq 0.001$) identified between early and late instars.	196
Appendix C Supplementary material for Chapter Six	197
Figure C6.1 ClustalOmega generated alignment of OAR transcript isoforms retrieved from <i>D. arcuata</i> transcriptome GIKL000000000.	200
Appendix D Supplementary material for Chapter Seven.....	201
Table D7.1 Summary of repeat content in <i>D. arcuata</i> genome generated by RepeatMasker using the arthropod repeat database.	201
File D7.1 Fasta sequences of scaffolds assembled by MaSuRCA and AGOUTI. .	202
File D7.2 Fasta sequences of <i>D. arcuata</i> putative proteins identified by WQ-MAKER.	202
File D7.3 Structural and functional annotations of <i>D. arcuata</i> putative genes identified by WQ-MAKER.	202

Chapter 1: General Introduction

Excerpts of this chapter have been adapted from the following:

Yack, J.E., & **Yadav, C.** (2021). Vibratory sensing and communication in caterpillars. In P. Hill (Ed.), *Biotremology: Physiology, Ecology, and Evolution* (In Press)

Statement of contribution:

All parts of this chapter were written by C. Yadav.

1.1 General overview

Social behaviour in animals is a widely studied topic, covering a broad range of behaviours such as parental care, the complex relationships of eusocial insects, and animal mating strategies (e.g. Alcock 2005; Costa 2006; Ward and Webster 2016). Studies on social behaviours can be broadly categorized into three types: a. descriptions of phenotypic traits (e.g. morphological and behavioural characteristics) associated with sociality; b. those focusing on the adaptive significance of social interactions, referred to as the ‘ultimate’ causations of behaviour; and c. those that focus on the sensory, physiological and genetic mechanisms underlying the behaviour, referred to as the ‘proximate’ causations of behaviour. My Ph.D. research focuses on the proximate mechanisms underlying social behaviour, and specifically group formation in larval Lepidoptera (caterpillars). While grouping behaviour is widespread and crucial to the survival of caterpillars (Costa 2006), there are few studies exploring the mechanisms used to form and maintain groups (e.g. Colasurdo and Despland 2005; Pescador-Rubio et al. 2011). I will venture into two relatively unexplored areas of study for larval Lepidoptera and for juvenile insects in general, by testing hypotheses on the roles of vibroacoustic communication and gene expression in mediating group formation.

My Ph.D. thesis has three main components: First, I will document the morphological and behavioural changes that occur during development of a caterpillar species that transitions from group living to solitary living; Second, I will experimentally test the roles of vibroacoustics in group formation; Third, I will explore the role of genes in mediating social behaviour by examining biases in gene expression over the course of development,

and then test their function by experimental manipulation. The remainder of this introduction provides a brief overview of social behaviour in general, with an emphasis on mechanisms of group living in insects, followed by the specific goals of my research, and how these goals are addressed in the respective thesis chapters.

1.2 Social behaviour defined

There are many definitions for what constitutes a social animal, or a social behaviour, and there has been much debate on the topic. There is a lack of proper characterization of social complexity, resulting in varying/incoherent definitions across organisms (e.g. Costa and Fitzgerald 2005; Costa 2006; Kappeler 2019). In social insects, the classification of sociality has been primarily based upon three key features—presence of overlapping generations, reproductive altruism and cooperative brood care, and insects exhibiting all of these features (e.g. honeybees, ants, termites) are considered “eusocial” (Batra 1966, Wilson 1971; Costa and Fitzgerald 2005). On the other hand, the presence of one or two of these features has been used for classification of species as solitary, subsocial, communal, quasisocial, and semisocial (Costa 1997; Costa and Fitzgerald 2005; Dew et al. 2016). Despite the continued use of this terminology for invertebrate species, there is no general consensus on a common definition of sociality for all species. As stated by Costa & Fitzgerald (2005) “we have simply agreed to disagree” over the context of several definitions for sociality provided by researchers over the years. In caterpillars, group-living behaviour has been termed as “communal” based on the traditional classification of sociality, where individuals from the same generation live together and no overlapping generations are present (Costa 1997). However, Costa (1997) argued that sociality in

caterpillars is much more complex than just the communal aggregation of individuals. In fact, eastern tent caterpillars are a great example for this, as the social larvae exhibit several group behaviours such as thermoregulation, cooperative foraging, defense, and shelter construction. Such behaviours indicate coordination among individuals in groups, which requires communication that can be achieved by different means such as chemical and acoustic communication, leading to the consideration that communication is the key basis of sociality (Costa 2006, 2010).

1.3 Ultimate and proximate mechanisms of social behaviour

Tinbergen's (1963) approach to studying behaviour, by exploring the ultimate functions and proximate mechanisms, has been widely applied to understanding social behaviours in insects (Jandt and Toth 2015; Kapheim 2018). Ultimate explanations provide us with insights into why insects exhibit social behaviours (i.e. the adaptive significance of performing social interactions). These include protection against predators, disease resistance, communal shelter and nest building, overcoming host plant structural defences, parental care, and improved efficiency through division of labor. Such benefits have been widely documented (e.g. Fitzgerald 1993; Choe and Crespi 1997; Fitzgerald and Costa 1999; Costa 2006; Korb 2009; Cremer et al. 2018). Proximate explanations for social behaviour include mechanisms such as sensory, genetic and neuroendocrine factors that might influence, for example, how individuals locate each other, form and maintain social groups, and interact with group members. The first step to exploring proximate mechanisms of social behaviour is to document the behaviour itself, and then to identify the mechanism(s). For example, temporal polyethism, the age-related changes in behaviour

in honeybee workers, has been widely studied with respect to mechanisms. These include changes in brain anatomy (Robinson and Vargo 1997), physiological and hormonal changes (Robinson 1992), and changes in gene expression in the brain (Ben-Shahar et al. 2002; Ben-Shahar et al. 2003; Whitfield et al. 2003). While such mechanisms have been widely studied in eusocial insects (e.g. Miura 2004; Jandt and Toth 2015; Zayed and Kent 2015; Leonhardt et al. 2016; Kamhi et al. 2017; Kapheim 2018), there are relatively few examples from group-living larval insects, including caterpillars. Therefore, in this thesis I focus on proximate mechanisms mediating social behaviour in caterpillars, exploring vibroacoustics and genomics.

1.4 Vibroacoustics and the role of vibrations in grouping behaviour

The importance of vibratory sensing and communication is progressively being recognized in insects, including juveniles (e.g. Cokl and Virant-Doberlet 2003; Cocroft and Rodriguez 2005; Fletcher 2007, 2008; Hill 2008; Yack 2016; Endo et al. 2019). Insects use vibrations in varied contexts. These include, for example, detection of passive cues (from abiotic or biotic sources), reproduction, territorial displays, recruitment, and other social interactions (Virant-Doberlet and Cokl 2004; Cocroft and Rodriguez 2005; Hill 2008; Cocroft and Hamel 2010; Yack 2016; Virant-Doberlet et al. 2019). The majority of these studies are focused on adults (mainly reproductive behaviours), with a limited number of studies in juveniles (eggs, larvae, nymphs and pupae). Yet, emerging studies suggest the use of vibrations by juveniles in diverse contexts; for example, egg hatching (Endo et al. 2019), social group coordination and recruitment (Hograefe 1984; Fletcher 2007, 2008; also see Chapter 4), parasitic or mutualistic interactions with ants (Devries 1990; Travassos and

Pierce 2000), food acquisition (Ishay 1974; Hograefe 1984; McIver and Beech 1986), territoriality and spacing (Yack et al. 2001, 2014; Fletcher et al. 2006; Scott et al. 2010; Guedes et al. 2012), and predator and parasitoid detection and avoidance (Castellanos and Barbosa 2006; Low 2008; Gish et al. 2012; Kojima et al. 2012). In this thesis I will explore the role of vibroacoustics in sociality in caterpillars.

In caterpillars, group-living is observed in >300 species spanning across 27 families (Costa and Pierce 1997; Costa 2006), and both ultimate and proximate explanations have been explored to understand the grouping behaviour. Ultimate explanations unravel why caterpillars form groups and these include enhanced survival (Stamp 1980; Fitzgerald 1993; Costa and Pierce 1997), improved foraging efficiency (Fitzgerald 1993; Ward and Webster 2016; Despland 2019), thermoregulation (Joos et al. 1988; Ruf and Fiedler 2002), and predator defense (Costa 1997; Costa and Pierce 1997). Proximate explanations, on the other hand, elucidate how caterpillars form and maintain groups, including the social interactions among individuals. Proximate mechanisms related to sociality studied in caterpillars to date mostly include chemical communication (Costa and Pierce 1997; Colasurdo and Despland 2005), or tactile communication (Fitzgerald 2003). Vibratory communication is increasingly being reported in caterpillars (e.g. Yack et al. 2001; Scott et al. 2010 a, b; Scott 2012), however, there are no examples examining its role in social grouping. Therefore, in my Ph.D. research I investigated vibroacoustics as a potential mechanism of social grouping in the masked birch caterpillar, *D. arcuata*, that exhibits group-living in the early stages of development, where vibratory signalling has been reported (Yack et al. 2001; Matheson 2011).

1.5 Sociogenomics and the genomic influences on grouping behaviour

Genetic influences on insect sociality are increasingly being explored because of the advent of genomic tools and resources over the last couple of decades. The emerging field of sociogenomics investigates which genes and pathways regulate those aspects of development, physiology and behaviour that influence sociality. One approach to studying the genetic regulation of social behaviours is to conduct comparative transcriptomic analyses to identify changes in gene expression associated with sociality (forward genomics), followed by testing the function of a specific gene in regulating behaviour by using gene silencing techniques such as RNAi. Comparative transcriptomic studies can be conducted either within or between species. Comparative analyses between species can help us to understand the evolution of sociality and conservation of gene function across species (Berens et al. 2014; Kapheim 2016, 2018). On the other hand, within species comparative studies can inform us on how the differential regulation of the same gene(s) can mediate social behavioural phenotypes within an organism (Kucharski and Maleszka 2002; Chen et al. 2010; Kapheim 2016, 2018). Once genes associated with sociality are identified by transcriptomics analyses, their function(s) can be further tested experimentally by manipulating their expression (e.g. by RNAi, CRISPR) and studying the effects on social behaviour. Despite the successful application of genomic studies to understanding the molecular basis of sociality in eusocial insect societies, there is a dearth of such studies in understanding social grouping behaviours in caterpillars. Therefore, using the masked birch caterpillar, *D. arcuata*, I will conduct comparative transcriptomics

analyses within a species, with a further preliminary gene function testing using RNAi to explore the genomic influences on social grouping in caterpillars.

1.6 Study organism: The masked birch caterpillar, *Drepana arcuata*

To explore the vibratory and genomic mechanisms mediating social grouping in caterpillars, I used the masked birch caterpillar, *Drepana arcuata* (Lepidoptera: Drepanoidea), as the study organism. This is primarily because of three reasons:

- (i) Previously, *D. arcuata* larvae have been noted to generate a complex repertoire of vibratory signals, using them in the solitary late instar stage during territorial encounters but also in earlier instars that live in groups (Yack et al. 2001; Scott et al. 2010a; Matheson 2011).
- (ii) *Drepana arcuata* larvae have been noted to transition from social to solitary behaviours during development, thus providing an excellent opportunity to conduct comparative transcriptomic analysis within a species (Matheson 2011).
- (iii) Different species of larvae belonging to the superfamily Drepanoidea exhibit varying levels of sociality, as well as vibratory signalling (Scott et al. 2010a; Scott 2012), making this group ideal to conduct comparative studies between species to investigate the mechanisms of sociality. In my Ph.D. research I will not be directly exploring inter-species comparisons, but will be setting the stage for such comparative investigations in the future.

1.7 Major thesis goals

The main objectives, hypotheses and specific goals of this thesis are summarized below.

Objective 1: Testing the role of plant-borne vibrations in group formation

I test the hypothesis that caterpillars use plant-borne vibrations to form social groups, using *D. arcuata* (Drepanidae, Drepanoidea) as a model species.

Specific Goals: **Goal 1** documents phenotypic changes (morphology, behaviour, acoustic signalling) throughout larval development using microscopy, laser vibrometry and videography. These are reported in Chapters 2 and 3. **Goal 2** tests the hypothesis that early instars (I-II) use complex vibratory signals to attract conspecifics by correlating vibratory signalling to group formation events. This is reported in Chapter 4.

Objective 2: A sociogenomics approach to understanding grouping behaviours

I test the hypothesis that differential gene expression mediates social and solitary behavioural states in caterpillars.

Specific Goals: **Goal 1** conducts a larval transcriptome analysis to describe differential expression of genes between social and solitary stages. These goals are addressed in Chapter 5. **Goal 2** tests the function of candidate “social” genes by altering target gene expression using RNAi. This is reported in Chapter 6. **Goal 3** assembles and annotates a draft genome for *D. arcuata*. This goal is addressed in Chapter 7.

Chapter 2: Immature stages of the masked birch caterpillar, *Drepana arcuata* (Lepidoptera: Drepanidae) with comments on feeding and shelter building

This chapter is an adaptation of the following publication:

Yadav, C., Yack J.E. (2018) Immature stages of the masked birch caterpillar, *Drepana arcuata* (Lepidoptera: Drepanidae) with comments on feeding and shelter building. *Journal of Insect Science*, 18(1), 1–9.

Statement of contributions:

C. Yadav collected and analyzed the data, prepared figures and wrote the paper; J.E. Yack supervised the project, helped with data analysis, contributed photographs, and editing.

2.1 Abstract

The masked birch caterpillar, *Drepana arcuata* (Lepidoptera: Drepanidae) is an excellent model for studying vibratory communication and sociality in larval insects. Vibratory communication occurs throughout development, but the functions of signals are reported to change as larvae change from gregarious to solitary lifestyles. To better understand the sensory ecology of these caterpillars, it is important to study their life history. Here, we describe the morphological and behavioural characteristics of larvae by confirming the number of instars, identifying their distinguishing morphological features, and noting changes in feeding and shelter construction. Five instars were confirmed based on the number of head capsules collected for individuals throughout development, and by using Dyar's rule, which predicts the number of instars based on geometric growth patterns of head capsules. Frequency distributions of head capsule widths showed five separate peaks, indicating that this is a useful parameter for distinguishing between instars. Other morphological features including body length, shape, and banding patterns of head capsules, and morphology of thoracic verrucae are helpful in distinguishing among instars. Feeding behaviour changes from leaf skeletonization in first and second instars to leaf cutting in fourth and fifth instars, with third instars transitioning between these feeding styles as they grow. Early instars typically construct communal silken shelters whereas late instars live solitarily in leaf shelters. These results provide essential life history information on the masked birch caterpillar that will enable future investigations on the proximate and ultimate mechanisms associated with social behaviour and communication in larval insects.

2.2 Introduction

Larvae of the arched hooktip moth, *Drepana arcuata* Walker (Lepidoptera: Drepanidae) have been studied as a model for larval vibratory communication, a poorly understood mode of communication in juvenile insects (Yack 2016). Late instars are territorial and generate ritualized acoustic signals to defend leaf shelters from conspecifics (Yack et al. 2001, 2014; Scott et al. 2010; Guedes et al. 2012). Early instars, unlike late instars, are reported to live in small groups and a recent study demonstrates that vibrational signalling is associated with recruitment of conspecifics (Yadav et al. 2017 [see Chapter 4]). In addition, vibration signals are proposed to function in other social interactions among early instars living in groups (Matheson 2011). The masked birch caterpillar (adults are referred to as arched hooktip moths) offers great potential for studying the roles of vibratory communication and mechanisms mediating social interactions in larval insects. To proceed with such investigations, it is important to be able to identify the larval stages, and to understand how life history traits change with each stage.

Drepana arcuata is broadly distributed throughout northern and east-southeastern North America (Rose and Lindquist 1997). Host plants of *D. arcuata* include *Betula papyrifera* Marshall (Fagales: Betulaceae), *Betula populifolia* Marshall (Fagales: Betulaceae), *Betula glandulosa* Michx. (Fagales: Betulaceae), *Betula alleghaniensis* Britton (Fagales: Betulaceae), *Alnus rubra* Bong. (Fagales: Betulaceae), and *Alnus rugosa* (incana) (L.) Moench (Fagales: Betulaceae) (Handfield 1999). Previous studies have reported on various life history, morphological, behavioural, and physiological traits (Packard 1890; Dyar 1895; Beutenmuller 1898; Stehr 1987). Adults are medium-sized, broad-winged with

hooked tips on the forewings (Figure 2.1). They possess abdominal ears that are ultrasound-sensitive and proposed to function in bat detection (Surlykke et al. 2003). Previous reports on immature stages provide mostly anecdotal details on the morphology or behaviour of late instars (sometimes referred to as ‘mature’ larvae) (e.g. Dyar 1895; Beutenmuller 1898; Stehr 1987). A more detailed study by Packard (1890) provides morphological descriptions of larval stages, but this study was limited in that it followed only a few, unspecified numbers of individuals and did not provide objective or quantifiable measures for distinguishing between larval stages. Furthermore, Packard (1890) provided limited information on instar-specific behaviours, and for only certain instars. Previous studies focusing on vibratory communication (Yack et al. 2001, 2014; Scott et al. 2010; Matheson 2011; Guedes et al. 2012; Yadav et al. 2017 [see Chapter 4]) described some characteristics of leaf shelters, conspecific communication, predator detection, and morphological features associated with signal production in unidentified ‘early’ or ‘late’ instars. Currently, there are no formal studies documenting instar-specific morphological and behavioural traits. The goals of this study are to document the number of instars, identify morphological criteria for distinguishing between instars and to note stage-specific behaviours associated with sociality including feeding, grouping, and shelter building.

Figure 2.1 Adult moth *D. arcuata* in resting position on a birch leaf. Scale bar: 5 mm.

2.3 Methods

2.3.1 Insect collection and rearing

Drepana arcuata (Lepidoptera: Drepanidae) were collected as moths from ultraviolet lights at the Queen's University Biology Station (Chaffey's Lock, ON, Canada, 44.5788°N, 76.3195°W) and a few other locations close to Ottawa, Ontario, Canada (45.4215° N, 75.6972°W) between May and September, 2010–2015. Gravid females were held in glass jars where they oviposited on paper birch (*B. papyrifera*) cuttings or brown paper bag clippings. Using a fine paint brush, neonates were transferred to fresh birch cuttings held in plastic vials and reared indoors at room temperature (21–23°C and 16 h: 8 h light:dark).

To determine the number of instars, neonate larvae were followed throughout their development. On the day of hatching, four to six neonates (first instars, 59 in total and obtained from >10 females) were transferred to leaves contained in a polystyrene petri dish (Falcon, 100 x 15 mm) (number of petri dishes = 14) lined with moistened paper towels. Larvae of the same age (i.e., hatched within 12 h of each other) were placed in petri dishes in small groups. First instars were kept in petri dishes instead of jars to facilitate collection of their very small head capsules. After molting to second instar, larvae were transferred to twigs of paper birch with 5–10 leaves. Birch twigs were inserted into the lids of water-filled plastic vials and care was taken to seal the bases of the twigs using reusable adhesive putty (Staples) to prevent wandering larvae from drowning. Each twig, in turn, was enclosed in large glass jars (23.5 x 14 cm) lined with moistened paper towels (number of jars = 10). Early instars were reared in small groups of 4–6 because of their lower survivorship when reared individually (personal observation, J. E. Yack). Jars and petri

dishes were examined daily to collect head capsules for subsequent measurements, to take photographs, to monitor feeding and shelter building activities, and to refresh food supplies. This enabled us to keep track of molting and keep track of head capsules for each individual caterpillar. A few individuals of each instar were preserved in 75% ethanol.

2.3.2 *Morphology*

Morphological features were assessed from live individuals, ethanol preserved specimens and from shed head capsules. Each live individual was examined at least once within 24 h of molting, and the final (fifth) instar was examined for an additional time period to document the prepupal stage. A number of morphological features, including color, setae, verrucae, and body length were recorded from live larvae in their natural resting positions on leaves. Shed head capsules were measured across the widest part for each larval stage (Dyar 1890) for instars I–IV. Because head capsules were deformed following ecdysis from fifth instar, these measurements were taken directly from live larvae on days 3–4 of the fifth instar.

Photographs were taken using a stereomicroscope (Leica M205 C, Leica, Wetzlar, Germany) equipped with a camera (Leica DMC4500, Leica, Wetzlar, Germany). Measurements, z-stacked images, and videos were obtained using Leica application suite V 4.2. A Nikon Coolpix camera (4500, Nikon, Japan) was used to obtain images of eggs, pupae, and adults. For scanning electron micrographs, head capsules were air-dried and mounted on aluminum stubs, sputter-coated with gold-palladium, and examined using a Tesca Vega-II XMU scanning electron microscope (XMU VPSEM; Brno, Czech

Republic). Identification and naming of various morphological traits followed the nomenclature of Stehr (1987) and Scoble (1992).

2.3.3 Behavioural observations

Behaviours were monitored and documented with photographs and videotapes at various times following 24 h after the molt for each instar. Feeding style was noted as being either by skeletonization (feeding only on the green tissue between the leaf veins) or cutting (whereby the mandibles cut through the full leaf). Shelter construction behaviours, including the patterns of silk deposition and location on the leaf, were recorded. Records were also made on each instar's tendency to live solitarily or in groups. However, because the nature of the rearing process (designed to follow individuals to collect head capsules) may have impeded the caterpillars' natural tendencies to group, a separate study on instar-specific grouping behaviours would be required.

2.3.4 Measurements to distinguish larval stages

We measured head capsule widths and body lengths of larvae in each instar in order to confirm the total number of instars and to distinguish between larval stages. To confirm the number of instars, in addition to counting the number of shed head capsules by following individuals, we also used Dyar's rule (Dyar 1890; Gaines and Campbell 1935; Cazado et al. 2014). Dyar's growth ratio was calculated by dividing mean head capsule width of one instar by the mean head capsule width of the preceding instar and then calculating the average growth ratio for all instars. We plotted the natural log of the mean head capsule width for each instar against the number of instars and conducted linear

regression analysis to determine if larvae follow a regular geometric growth progression (Dyar 1890; Gaines and Campbell 1935). To identify morphological traits for distinguishing between larval stages, we plotted both normal distribution values of head capsule widths and body lengths (within 24 h of molting from preceding instar) of larvae in each instar and conducted analysis of variance (ANOVA) by using one-way ANOVA tests followed by Tukey's HSD in R Studio 1.0.136 (R Core Team 2016) package Agricolae (de Mendiburu 2016). All the statistical analyses in this study were performed at $P < 0.05$.

2.4 Results

2.4.1 General comments on immature stages and rearing

Our results confirm that *D. arcuata* has five larval instars. Twenty two individuals were followed from neonatal to pupal stages. More data were collected for early than late instars for two reasons (1) higher mortality was observed in early instars (25%) and; (2) 1–3 representatives for each instar were preserved in 75% ethanol for reference (Instar I, II, III = 3 larvae of each, IV, V = 2 larvae of each). Development time from hatching to pupation took 16–22 d (mean = 19.32 ± 1.73 , $n = 22$).

2.4.2 Eggs

Morphology

Eggs were smooth, polished, flattened and oval (Figure 2.2). Diameter: 0.64–0.84 mm (mean = 0.77 ± 0.03 mm, $n = 46$). Adults laid eggs in rows of 2–14 on both the upper and lower surfaces of leaves, as well as on the plant stems, paper bag clippings, and the sides

of the glass jars. The color of fertilized eggs changed from yellow when laid, to orange-brown, reddish-brown, and then to black as they neared hatching. It took approximately 9–11 d for eggs to hatch. Neonates hatched at different times, with the exit holes about one-third diameter of the eggs shells, and oriented away from adjacent eggs.

Figure 2.2 Eggs of *D. arcuata* laid in rows on birch leaves. (A) One day old yellow eggs (left) and 3 days old light orange colored eggs (right) laid on the upper surface of a birch leaf. Scale bar: 4 mm; (B) Nine days old dark brown-black colored eggs laid on the underside of a birch leaf. Scale bar: 4mm. (C) Hatched eggs showing exit holes. Scale bar: 500 μm .

2.4.3 *First instar*

Morphology

Head capsule width: 0.26–0.31 mm (mean = 0.29 ± 0.01 mm, $n = 42$), body length: 1.75–2.93 mm (mean = 2.36 ± 0.33 mm, $n = 42$) (Figures 2.3 and 2.4; Tables 2.1 and 2.2). Head capsule black, shiny, granulated, triangular, rounded on top with a slight notch at the dorsal end of epicranial suture; head is approximately the same width or slightly wider than the body. Body mostly dark brown black with bright pale colored prothoracic segment (T1) and abdominal segments A1, A7; two dark brownish black parallel lines on the subdorsal surface running along the segments from T1 to A10. Pairs of brown-black dorsal and subdorsal structures called tubercles by Packard (1890) and verrucae by Stehr (1987) are present on thoracic (T1, T2, T3) and abdominal segments. We follow Stehr's term "verrucae" to provide descriptions of these setae. Verrucae on thoracic (T1, T2, T3) and abdominal segments (A9, A10) are slightly more prominent than those on other segments. The subdorsal verrucae on T1 are the most prominent. Dorsal verrucae on thoracic (T2, T3) segments bear one seta whereas the subdorsal verrucae bear two setae. Greyish-black, elongated thoracic legs are present on T1–T3; 4 pairs of light brown, thick, rounded abdominal prolegs are present on A3–A6, and the anal proleg on A10 is absent. Clear, forked setae arise from the dorsal and lateral surfaces on all thoracic and abdominal segments. A uranal plate is formed by the last abdominal segment (A10), and has a small, brownish, conical, bifurcated projection (called a "knob" or "process" by Stehr 1987), is covered with very short bristles, and two long setae emerging from the bifurcated tip. Developmental time (mean $\pm$ SD, $n = 42$) = 4.36 ± 0.90 d.

Feeding and shelter construction

Post hatching, neonate larvae wander individually until they find a location to feed and build a shelter, eventually forming small groups. Larvae construct a tent-like silk shelter by first laying a silk mat on either the upper or lower leaf surface, followed by spinning silk threads, slightly folding the leaf edge. Larvae begin constructing the shelter by first spinning two silk strands on either side of the shelter which they then extend into multiple cell-like units by attaching smaller silk strands with slight webbing. The location of the shelter is variable, with most shelters (~85%) formed at the edges of leaf. The size of the shelter also varies depending on the size of group contributing to shelter construction (e.g., 0.7 cm for a group of two individuals to 2 cm with seven individuals). First instars typically make only one shelter during this stage, and molt within the same shelter. Shelter building activity alternates with resting, walking, and feeding behaviours within the shelter. Larvae attach frass to the silk canopy of the shelter (Figure 2.4B). Larvae skeletonize the leaf surface within the shelter, with feeding spots of variable size and number depending on the number of individuals residing within the shelter. When several larvae reside together in shelters they often, but not always, work on edges of the same feeding spot. They extend both the feeding spots and shelters as they skeletonize the leaf tissue. Larvae tended to form small groups within 24 h of placing them in petri dishes.

Figure 2.3 Light micrographs (left) and scanning electron micrographs (right) of *D. arcuata* head capsules: (A) first instar. Scale bar: 100 μm ; (B) second instar. Scale bar: 200 μm ; (C) third instar. Scale bar: 300 μm ; (D) fourth instar. Scale bar: 500 μm (E) fifth instar. Scale bar: 500 μm .

Figure 2.4 Lateral (left) views of each instar and dorsal views of larvae within their characteristic shelter (right). (A) First instar lateral view. Scale bar: 500 μm ; (B) first instars in shelter. Scale bar: 2,000 μm ; (C) second instar lateral view. Scale bar: 1,000 μm ; (D) second instars in shelter. Scale bar: 2,000 μm ; (E) third instar lateral view. Scale bar: 2,000 μm ; (F) third instar in shelter. Scale bar: 3,000 μm ; (G) fourth instar lateral view. Scale bar: 2,000 μm ; (H) fourth instar in shelter. Scale bar: 3,000 μm ; (I) fifth instar lateral view. Scale bar 5,000 μm ; (J) fifth instar in shelter. Scale bar: 5,000 μm .

Table 2.1 Head capsule measurements for instars (I–V) of *D. arcuata*.

Instar	N	Range (mm)	Mean $\pm$ SD (mm)	Coefficient of variation	Dyar's ratio*
I	42	0.26-0.31	0.29 $\pm$ 0.01	0.04	---
II	32	0.46-0.56	0.52 $\pm$ 0.03	0.05	1.79
III	28	0.77-1.02	0.92 $\pm$ 0.06	0.06	1.79
IV	25	1.20-1.75	1.46 $\pm$ 0.12	0.08	1.57
V	22	1.56-2.21	2.01 $\pm$ 0.18	0.09	1.40

*Dyar's ratio (e.g. for instar II): mean head capsule width of Instar II/Mean head capsule width of instar I

Table 2.2 Body length measurements for instars of *D. arcuata*.

Instar	N	Range	Coefficient of variation (mm)	Mean $\pm$ SD (mm)
I	42	1.75-2.93	0.14	2.36 $\pm$ 0.33
II	32	3.09-5.58	0.17	3.85 $\pm$ 0.67
III	28	4.20-7.50	0.16	5.72 $\pm$ 0.91
IV	25	6.62-10.60	0.13	8.50 $\pm$ 1.08
V	22	10.00-20.50	0.22	13.95 $\pm$ 3.01

2.4.4 *Second instar*

Morphology

Head capsule width: 0.46–0.56 mm (mean = 0.52 ± 0.03 mm, $n = 32$), body length: 3.09–5.58 mm (mean = 3.85 ± 0.67 mm, $n = 32$) (Figures 2.3 and 2.4; Tables 2.1 and 2.2). Head capsule differs from the first instar primarily in color and banding pattern, with the color becoming lighter brown and the appearance of two, not so well pronounced, dark brown transverse bands across the head. Overall, the body color is lighter brown but with the same color pattern as first instars. Lateral verrucae on thoracic segment T1 similar in size to the dorsal and subdorsal verrucae on thoracic segments T2 and T3; dorsal and subdorsal verrucae are present on T2, T3, and lateral verrucae on abdominal segment A9 are slightly more prominent and conspicuous than in the first instar. Bifurcation of the conical projection at the end of the suranal plate is less well pronounced than in first instars, covered with more conspicuous black setae. A pair of setae, emerging from the bifurcation, is shorter than the projection itself. Developmental time (mean $\pm$ SD, $n = 32$) = 4.21 ± 0.64 d.

Feeding and shelter construction

Following the molt from first instar, the exoskeleton is consumed in most cases (in >90% of the cases) while the head capsule is often attached to the overhanging strands of the silk shelter (in >80% of the cases), or present on the floor of silk shelter (<20% of the cases). Second instars either continue extending the same first instar shelter or make a new shelter on the same leaf. Feeding and shelter construction behaviours are similar to those observed in the first instar.

2.4.5 *Third instar*

Morphology

Head capsule width: 0.77–1.02 mm (mean = 0.92 ± 0.06 mm, $n = 28$), body length: 4.20–7.50 mm (mean = 5.72 ± 0.91 mm, $n = 28$) (Figures 2.3 and 2.4; Tables 2.1 and 2.2). Head capsule differs from the second instar in shape, color, and banding pattern; the shape becomes slightly round, the color is yellowish-green, and there are two clear, brown transverse bands across the head. Overall, the body color changes to yellowish green with a similar pattern as previous instar. Lateral verrucae on T1 are reduced in size compared to dorsal and subdorsal verrucae on T2 and T3; dorsal and subdorsal verrucae on thoracic segments T2, T3, as well as other verrucae on abdominal segments, are much more prominent than in the second instar; verrucae on abdominal segments are lighter yellow to red. Thoracic legs are blackish-brown, and abdominal prolegs are yellowish-brown to green. The conical projection at the end of suranal plate is rust colored with a black tip and more conspicuous black setae. Developmental time (mean $\pm$ SD, $n = 28$) = 3.75 ± 0.81 d.

Feeding and shelter construction

Following the second instar molt, third instars continue to feed within the same shelter by skeletonizing the leaf tissue, but then transition to cutting the leaf edges, moving inwards and consuming silk strands as they feed. Within 24 h of molting, third instars move out of the early instar shelter and make a new solitary shelter on the edge or tip of a leaf. Leaf skeletonization is no longer observed after this point in their development. Shelter construction involves more folding of the leaf compared to early instars with occasional

attachment of frass to the overhanging silk strands. Shelters consist of thicker silk strands joining one end of leaf to another, thus folding the leaf more so than in earlier instars. Prior to molting into fourth instar, third instars construct a “premolting” shelter that can either be a new shelter or a modification of the existing shelter. The premolting shelter comprises additional layers of silk, making it denser.

2.4.6 *Fourth instar*

Morphology

Head capsule width: 1.20–1.75 mm (mean = 1.46 ± 0.12 , $n = 25$), body length: 6.62–10.60 mm (mean = 8.50 ± 1.08 , $n = 25$) (Figures 2.3 and 2.4; Tables 2.1 and 2.2). Head capsule differs from previous instar mainly with respect to shape, becoming completely round, and bilobed with a prominent, well-defined notch at the epicranial suture. Overall body color changes to lighter green, with a green thorax and abdominal segments green mottled with brown spots; the pattern is the same as the preceding instar. Lateral verrucae on T1 reduced in size, becoming flat; dorsal and subdorsal verrucae on T2, T3 are more conspicuous and prominent than others on abdominal segments and compared to the verrucae on T2, T3 of the previous instar; dorsal verrucae on T2, T3 are bigger than subdorsal ones with a yellowish-green base and black tip; distinctly visible oval, green spiracles with brown outline present on T1, A1–A8. Thoracic legs and abdominal prolegs are green. The conical projection at the end of A10 is bright rust-red colored with a small pair of setae emerging from the significantly reduced bifurcation. Developmental time (mean $\pm$ SD, $n = 25$) = 3.55 ± 0.80 d.

Feeding and shelter construction

Larvae lay a silk mat on the leaf surface as do previous instars but while constructing the shelter fold the leaf significantly more so than third instars. Fourth instars also construct feeding and molting shelters as described for third instars. Larvae may make multiple shelters for resting and feeding that consist of only a few (2–4) thick silk strands with no cells and webbing. Some larvae were observed to remove frass from the shelter by either backing up and flicking over the edge of the leaf, or by picking it up in their mandibles, walking to the leaf edge and dropping it. Like late third instars, fourth instars feed by cutting the leaf and consuming the shelter as they feed. Once the larva consumes the entire leaf, it wanders in search of a new leaf to construct a shelter.

2.4.7 Fifth instar

Morphology

Head capsule width: 1.56–2.21 mm (mean = 2.01 ± 0.18 mm, $n = 22$), body length: 10.00–20.50 mm (mean = 13.95 ± 3.01 mm, $n = 22$) (Figures 2.3–2.5; Tables 2.1 and 2.2). Morphology and color patterns of head capsules and body do not change significantly from fourth instar; dorsal abdominal body color turns rust-brown. Dorsal and subdorsal verrucae on thoracic segments T2, T3 more prominent than other verrucae on the body and compared to T2, T3 thoracic verrucae on the previous instar; dorsal verrucae almost double the size of subdorsal ones with a yellow base and bright red tip; lateral verrucae on A9 green and inconspicuous. Prepupal larvae become enlarged, with the head capsule noticeably more narrow than the body; dorsal surface of abdominal segments turns

brownish-red with green thoracic segments; prominent verrucae are only present on thoracic segments T2, T3. Developmental time (mean $\pm$ SD, n = 22) = 4.41 $\pm$ 0.80 d.

Feeding and shelter construction

Same as observed in fourth instar. Prior to pupation, larvae may wander and eat multiple leaves before settling into a silk shelter that they construct specifically for pupation. A fifth instar feeds for 2–3 d before entering prepupal stage and then continues feeding for 1–2 d before folding the leaf. Once the leaf is entirely folded, it takes 2–3 d for the pupa to form. This shelter consists of several (~5–10) thick silk strands that fold the leaf edge tightly, encasing the pupa.

2.4.8 Pupa

Morphology

Length: 11.19–13.84 mm (mean = 12.49 $\pm$ 0.88, n = 10); width: 3.67–4.40 mm (mean = 3.98 $\pm$ 0.30, n = 10) (Figure 2.5). The pupa is medium to dark brown colored with fine hair-like setae present on abdominal segments. Darker brown to black colored spiracles are also present on the abdominal segments. Duration: the duration of the pupal stage is temperature dependent. We did not formally measure this, but in general, the duration is about 2 weeks at room temperature (~21–23 °C). Pupae can also successfully overwinter for over a year at 4–6 °C (J. E. Yack, unpublished observations).

Figure 2.5 Light micrographs of prepupa and pupa of *D. arcuata*. (A) Prepupal phase showing thick strands characteristic of pupal shelters. Scale bar: 7 mm. (B) As silk dries, it contracts forming a tight leaf enclosure for the pupa. Pupa is shown in the inset. Scale bar: 500 μm .

2.4.9 Comparisons between sizes of instars

Five head capsules were collected for each of the individuals followed throughout the larval development from hatching to pupation. When we plotted the distribution of head capsule widths using normalized values (Figure 2.6A), five distinct peaks were observed. Furthermore, head capsule widths of each instar were significantly different from each other at $P < 0.05$ (one-way ANOVA; $F_{4,144} = 1,797$, $P < 0.0001$ and Tukey's HSD test). This indicated that there are five distinct instars and head capsule width is a good indicator of instars. Furthermore, the natural log of head capsule widths plotted against instars showed geometric larval growth through development (Figure 7; Dyar's average growth ratio: 1.63) hence further confirming five instars for *D. arcuata*. Body lengths for larvae in each instar were also measured within 24 h of molting. Although we observed five peaks corresponding to five instars with each significantly different from each other (one-way ANOVA; $F_{4,144} = 328.8$, $P < 0.0001$ and Tukey's HSD test), as expected there was more overlap in lengths across instars with higher coefficients of variation for each instar than for head capsule widths (Tables 2.1 and 2.2; Figure 2.6B).

Figure 2.6 Distribution of head capsule widths and body lengths for *D. arcuata* instars. (A) Normalized distribution of head capsule widths; (B) normalized distribution of body lengths recorded at 24 h following molt for each instar.

Figure 2.7 Natural log of mean head capsule widths plotted against the number of instars. The figure shows a linear geometric progression ($R^2 = 0.98$, $P = 0.0005$), confirming five instars for *D. arcuata*.

2.5 Discussion

Two main goals of this study were to confirm the number of instars and to establish morphological criteria to distinguish between these instars. By following molts and collecting head capsules for multiple individuals, we confirmed that *D. arcuata* larvae undergo five instars. We further confirmed five instars using Dyar's rule (1890). Dyar showed a more or less constant, geometric progression in larval head capsule widths for 28 larval lepidopteran species. Dyar's rule (1890) has been used to identify the number of larval stages in several insects (e.g. McClellan and Logan 1994; Delbac et al. 2010; Velásquez and Vilorio 2010; Barrionuevo and San Blas 2016). Head capsule width is the most reliable way to identify a larval stage, as it does not change within an instar.

In addition to head capsule width and body length, a number of other morphological characteristics differed between instars. First instars differ from all others based on their characteristic black, triangular head, and dark brown body. Second instars develop faint brown bands on their heads with comparatively more prominent verrucae on thoracic and abdominal segments. Third instars are the first to develop two clear, distinct brown transverse bands on the head, with the head becoming more rounded than oval. Between third and fourth instar, the head becomes more rounded, thoracic legs and abdominal prolegs become green in color, and the overall body color becomes lighter green mottled with brown on abdominal segments. Aside from head capsule width and body size, the most noteworthy change from fourth to early fifth instar is the very prominent bright red-tipped dorsal verrucae on thoracic segments T2 and T3. However, as fifth instars reach the prepupal stage their dorsal abdominal surface becomes rust-brown, and the overall body

becomes enlarged with the head size almost half as wide as the body. While we did find consistency in body color within instars, this morphological feature should be further studied in larvae reared on different colored leaves, it has been noted that body color of late instars may vary depending on the leaf color (personal observation, J. E. Yack), and therefore this feature should be used cautiously in distinguishing between instars, and warrants further attention.

Our study is the first to document instar-specific behavioural changes in this species, focusing on feeding style and shelter construction. Feeding style changes from exclusively skeletonizing the leaf surface in first and second instars, to exclusively cutting the leaf in fourth and fifth instars. Third instars exhibit both behaviours, and transition from one to the other as they mature. Changes in feeding style from skeletonizing in early instars to cutting in late instars have been previously noted for a number of Lepidoptera (Hochuli 2001). These changes could be attributed to the size of the head and mandibles, and changing nutritional requirements (Hochuli 2001). We did not assess how late instars cut the leaf or how they processed the leaf material after cutting the edge (i.e., whether they snipped, crushed, or chewed the leaf material), as this would require further analysis of the mandible structure and gut contents (e.g. Bernays and Janzen 1988).

All instars were observed to lay a silk mat on the leaf surface in addition to building silk shelters. Silk mats in many Lepidoptera are suggested to help larvae feed efficiently on leaves with high trichome density (Fordyce and Agrawal 2001) and also to provide protection from predators as the larvae grip on the silk mat when attacked (McClure and

Despland 2011). All *D. arcuata* instars constructed intricately woven silk shelters for resting, molting, and feeding. Many lepidopteran larvae construct silk shelters (Stehr 1987; Scoble 1992), with potential benefits such as defense and improved microclimate (Hunter and Willmer 1989; Costa and Pierce 1997). Leaf folding, which was more pronounced in the solitary shelters of late instars could have been aided by contraction of drying silk strands (see Fitzgerald et al. 1991; Fitzgerald and Clark 1994). First, second, and occasionally third instars attached frass to silk shelters. Fourth and fifth instars were observed to remove frass from their shelters, possibly to eliminate olfactory cues to avoid being detected by predators and parasitoids (Weiss 2003). The number and types of shelters change as larvae mature and change from a gregarious to solitary lifestyle as seen in several other lepidopterans (e.g. Abarca et al. 2014). These changes could be attributed to differences required for shelter and protection from predators and parasitoids, or different feeding habits with increasing body size (Lind et al. 2001).

Our study shows that early instars (I, II) form small groups within silk shelters whereas late instars do not (IV, V), supporting previous observations in both the lab (Yack et al. 2014; Yadav et al. 2017 [see Chapter 4]) and field (J. E. Yack, unpublished observations). Post hatching, early instars (I, II) use vibrational signalling in the formation of small groups (Yadav et al. 2017 [see Chapter 4]), whereas in late instars (IV, V) vibrational signalling is used in territorial encounters (Yack et al. 2001; 2014; Guedes et al. 2012). It is still unknown how early instar groups are maintained, but vibratory signals likely play a role. Third instars exhibited both behaviours in that they resided in the communal shelter for a period following their molt, but within 24–48 h of molting became solitary. Gregariousness

is an important life history trait observed in a number of lepidopteran larvae with a large number of species living gregariously either throughout or through a part of their development (Costa and Pierce 1997; Costa 2006). However, the mechanisms mediating sociality in larval Lepidoptera requires further study. The masked birch caterpillar is an excellent model to investigate the multimodal mechanisms involved, as they clearly transition from being gregarious to solitary during development, produce vibratory signals throughout their development, while at the same time possibly using chemical cues associated with silk in their shelters. In particular, the interesting transitional features of third instars provide opportunities to study physiological and genetic mechanisms underlying social behaviour in larval insects.

Chapter 3: Developmental changes in social grouping and vibratory signalling in the masked birch caterpillar, *Drepana arcuata* (Drepanidae)

Statement of contributions:

C. Yadav collected and analyzed the data, prepared figures and wrote the paper; M. Naranjo Vera helped with signal characteristics analysis; L. Turchen helped with statistical analyses; J.E. Yack supervised the project, helped with data analysis, contributed photographs, and editing.

3.1 Abstract

The masked birch caterpillar, *Drepana arcuata*, has been previously reported to exhibit grouping behaviour and vibratory signalling. However, developmental changes in these behaviours have not been documented. In the current study, I investigated changes in the tendency to form groups, and perform vibratory signalling behaviours, as *D. arcuata* larvae grow from instar I-V. Group-formation trials revealed that instars I-III form groups, that varied in size (1-5 in instars I,II; 1-2 in instar III), whereas instars IV-V remain solitary. By analyzing laser vibrometer and video recordings of signalling caterpillars, we confirmed four signal types - buzz scrape (BS), anal scrape (AS), mandible drum (MD) and mandible scrape (MS). Furthermore, we identified that while instars I-III generate all the four signal types, late instars only generate three signal types (AS, MD, MS). Signal rate analysis between instars revealed that (a) instars I-III have higher overall signalling rates per individual than late instars (IV,V), regardless of their group size, with AS being the most common signal in all instars when measured in their natural conditions; (b) solitary individuals (i.e. group size=1) in instars I-III signal at higher rates than solitary late (IV,V) instars, with AS again being the most frequent signal; and (c) in early instars (I,II), solitary individuals (i.e. group size=1) signal more than individuals in larger groups. This research confirms that there are instar specific changes in both social and signalling behaviour. We pose hypotheses explaining how transitions in social behaviours may be mediated by concurrent changes in vibratory signalling behaviours. One of these hypotheses, that early instars use vibratory signalling for social recruitment, is formally tested in the following chapter (Chapter 4) of this thesis.

3.2 Introduction

Group living occurs during some or all developmental stages in larval insects belonging to a wide range of taxa (Costa and Pierce 1997; Costa 2006). While some larvae remain in groups throughout their larval development, others become solitary as they grow (Reader and Hochuli 2003; Costa 2006). Changes in grouping behaviour during larval development could be a consequence of increasing costs to benefits ratios of living in groups as the larvae develop (Despland and Hamzeh 2004). The ultimate benefits associated with living in groups have been studied in larvae of different insect orders including Lepidoptera, Hymenoptera and Coleoptera (Costa 2006; Costa et al. 2007; Weed 2010). These benefits include enhanced thermoregulation (Seymour 1974; Fletcher 2009; Bryant et al. 2010), predator defense (Sillén-Tullberg 1990; Vulinec 1990), and feeding facilitation (Denno and Benrey 1997). The proximate mechanisms mediating grouping behaviours, such as group formation and maintenance, are not well understood for most species. The processes involved in group formation and cohesion can be studied at many levels of analysis, including sensory and neural mechanisms, hormonal and genetic. One of the sensory mechanisms that is of particular interest is vibratory communication, as vibratory signalling is increasingly being reported in larval insects (e.g. Yack et al. 2001; Fletcher 2007, 2008; Scott et al. 2012; Yack and Yadav 2021). Although chemical communication has been widely explored as a mechanism for grouping in larval insects (Costa and Pierce 1997; Costa 2006; Despland and Santacruz-Endara 2016), vibrational communication is yet to be explored as a recruitment mechanism in most species (but see examples for sawfly and beetle larvae in Hoegraefe 1984; Fletcher 2007, 2008; Coccoft and Hamel 2010).

The masked birch caterpillar, *Drepana arcuata* (Lepidoptera: Drepanidae), is an interesting model to study vibratory mediated social interactions in larval insects, for two reasons. First, it has been previously noted that the larvae transition from social to solitary living throughout development (Matheson 2011) (Figure 3.1), thereby offering an opportunity to study proximate mechanisms mediating grouping or solitary behaviour within a species. Second, it has been reported that all developmental stages produce vibratory signals, although the role of these vibrations has only been experimentally investigated in solitary late instars, who use vibrations for territorial disputes (Yack et al. 2001, 2014; Guedes et al. 2012). A key first step in understanding whether vibratory communication could play a role in group formation or other social interactions in this species is to formally document (i) how instars differ in their group formation behaviours, and (ii) how vibratory signalling differs, if at all, between instars living in their natural conditions. Obtaining this information will then enable us to identify differences in signalling behaviours that are correlated with grouped vs. solitary states, leading to more refined hypotheses on how signalling may function in group formation and maintenance.

There are four main goals of this study, classified under two broad objectives. **Objective 1** is to test the hypothesis that grouping behaviour changes throughout larval development. Specific goals will be to study (i) differences in the tendency to form groups between larval instars, and (ii) differences in group size at particular time points between instars.

Objective 2 is to assess how vibratory signalling differs between larval instars and between grouped vs. solitary individuals. Specific goals will be to: (iii) identify/confirm the types of signals that *D. arcuata* larvae produce under natural conditions. In previous studies, up to four types of vibration signals were described in *D. arcuata* (see Yack et al. 2001, 2014;

Matheson 2011; Guedes et al. 2012). These are: anal scraping, buzz scraping, mandible scraping and mandible drumming. However, these studies either focused only on late instars, or did not formally document instar-specific differences in signalling. Therefore, I aimed to confirm the types of vibratory signals occurring across all instar stages based on video recordings of caterpillars producing vibrations, and vibration characteristics of these signal types; (iv) assess how vibratory signal types and rates differ between instars while larvae reside in either solitary or grouped conditions. Collectively, this information will allow us to determine if larval instars differ in their grouping behaviours, and how signal types and characteristics differ between instars. Should both of these be confirmed, we can pose hypotheses on how vibratory signalling may mediate various social interactions, to explore experimentally in future studies.

Figure 3.1 Early and late instar *D. arcuata* larvae. A. Group of second instars in a silk shelter. Scale bar= 2000 μm ; B. Solitary fifth instar in silk shelter. Scale bar=7500 μm ; C. Group of first instars. Scale bar= 2000 μm ; D. Solitary fourth instar feeding near shelter. Scale bar=6000 μm .

3.3 Methods

3.3.1 Study animals

Gravid female *Drepana arcuata* (Lepidoptera: Drepanidae) moths were collected at ultraviolet lights at Queen's University Biology Station (Chaffey's Lock, ON, Canada), Algonquin National Park (ON, Canada) and few other locations near Ottawa, ON, Canada between May and September, 2010-2018. Females oviposited on paper birch (*Betula papyrifera*) cuttings held in plastic vials filled with water, or brown paper bag clippings. Neonates were carefully transferred to fresh paper birch leaves on twigs held in water-filled plastic vials kept inside glass jars. Larvae were reared indoors at room temperature of 21-23°C and 16:8h light:dark cycle. When it was necessary to separate individuals according to their instar, following each molt, larvae were transferred to new jars, keeping individuals of specific instars within same jar. Individual instars were identified primarily by their head capsule widths (Yadav and Yack 2018 [see Chapter 2]). Depending on the information required for different goals, the larvae were set up differently. See the respective sections below for more details.

3.3.2 Group formation trials

The first objective of this study is to test the hypothesis that grouping behaviour changes throughout larval development. Goals **i** and **ii** were to document the differences in tendencies to form groups, as well as differences in group size at particular time points between instars, respectively. To achieve these goals, a total of 25 group formation trials (five experiments for each of the five instars [I-V]) were performed. In each trial, a birch sprig (10-13 cm long) with five leaves was placed in a water filled plastic vial (Figure 3.2).

Then, the five individuals were placed individually on each leaf within a ~60 s period. Larvae were then monitored for group formation at 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h and 24 h. At each time point, each birch sprig was monitored for the number of solitary or grouped shelters, as well as for the number of individuals in a group. Group refers to one or more larvae residing in an established silk shelter, and group size reflects the number of larvae that are present inside the shelter. A group was considered to be “established” based on the presence of a silk shelter and/or feeding site within, or in vicinity of the shelter. It was not possible to identify individuals throughout a trial, but during each time interval, it was assessed whether all original five individuals remained on the birch sprig. Trials were performed indoors at room temperature (21-24°C), and no larvae were reused during any of the trials.

Group formation data were analyzed for the proportion of individuals that formed groups over 24 h period out of total number of individuals used (i.e. 25 per instar; 5 per trial; total trials=5 per instar), as well as the number and size of groups formed. To represent group formation over time, the proportion of individuals residing in groups, at different time points (0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h and 24 h), were calculated. Also, group formation data were subjected to survival analysis to follow the progression of grouping over time by each instar. To do survival analysis, normality and homoscedasticity of the data obtained were checked using Shapiro-Wilk’s and Bartlett’s tests, respectively. The data of proportion of solitary larvae within each instar through time were subjected to survival analyses using Kaplan-Meier estimators with the package *survival*, and when significant the curves were

contrasted using the pairwise multiple comparison. All analyses were performed using the R-software version 3.5.1 (R Core Team 2019).

Figure 3.2 Set-up used for group formation trials conducted for *D. arcuata* instars I-V. One individual was placed on each leaf and observed group-formation over a period of 24 h. This figure shows 5th instar. Scale bar=30 mm.

3.3.3 Types of vibratory signals and variation within and between instars

Objective 2 was to assess how vibratory signalling differs between larval instars. The first goal for this objective (iii) was to identify/characterize the signal types that *D. arcuata* larvae produce based on video and vibration recordings. Once the signal types had been confirmed, the second goal was to assess how vibratory signalling rates differ between instars.

General Vibration and Video Recording Set up

Two types of vibration recordings were conducted, one without video and one with video. Vibration recordings without video were conducted using a laser-Doppler vibrometer (PVD-100, Polytec Inc., Irvine, CA, USA) (velocity 20mm/s; high pass filter off; low pass filter 20 kHz). The laser beam focused on a disc of reflective tape (2 mm diameter) attached to the upper leaf surface (perpendicular distance of ~30 cm from the laser) within 1-2 cm from the individual(s) in the shelter, and the output was connected to a Fostex FR2 (Gardena, CA, USA) recorder (sampling rate of 44.1 kHz). All recordings were performed in a sound proof acoustic chamber (Eckel Industries, Cambridge, MA, USA) on a 45cm x 45cm x 5cm (L x W x H) block of slate upheld by tennis balls to minimize extraneous vibrations. Digital files were subsequently analyzed for temporal and spectral characteristics of each signal type using RavenPro Bioacoustics Research program 1.4 (Cornell Laboratory of Ornithology, Ithaca, NY, USA) using 2048 hanning window. Details of the analyses are described further below. The second type of recording involved simultaneous vibration and video recordings with the output from the laser-Doppler vibrometer (same specs as above) connected to the microphone port of a camcorder (Sony

hd-rxr520v Handycam, Sony Corp., Tokyo, Japan). These files were analyzed for confirming the signal types generated by each instar, as well as to assess differences in signal rates between instars.

What types of signals do D. arcuata produce?

Goal **iii** was to confirm the types of signals produced by *D. arcuata* based on body movements that coincided with vibrations. Signal types were also differentiated based on their vibration characteristics. This was done by comparing signal types within individuals of same instar. This was necessary to avoid variation that may result between signal characteristics due to differences in body size, substrate, location from reflective disc etc. between individuals. To confirm the types of vibration signals in the *D. arcuata* repertoire, I first viewed over 6 hours of video footage in total with simultaneous laser recordings from all instars in different behavioural conditions (e.g. resting, feeding, shelter building, being joined by conspecifics) with approximately equal time recordings from each instar (see below). Larval body movements were carefully observed by watching the video recordings at reduced speed along with laser audio to identify the signal types. Signals were categorized using previous categories for *D. arcuata* noted by other authors (e.g. Yack et al. 2001; Matheson 2011) [i.e. anal scrape, buzz scrape, mandible drum and mandible scrape], and at the same time noting whether other types of signals were evident. Observations on other behaviours such as feeding, shelter construction, and crawling were also made in order to distinguish between vibrations resulting from signalling and those arising from other incidental activities.

Morphological components associated with signal types, namely PP1 (Posterior proctor seta 1) (c.f. Yack et al. (2001); Scott et al. (2012)), were obtained using light and scanning electron micrographs (SEMs) for each instar. For SEMs, larvae were air-dried and mounted on aluminum stubs, followed by sputter-coating with gold-palladium, and examination using a Tesca Vega-II XMU scanning electron microscope (XMU VPSEM; Brno, Czech Republic). To confirm the presence of anal oars, three to five individuals per instar were also observed using light microscopy. SEMs were then visually compared to identify differences between instars.

To establish whether signal types categorized by morphology and body movements also differed in their signal characteristics, vibrations from individuals that produced a variety of signal types were analyzed. This was done by analyzing .wav files in RavenPro1.4 for the duration, bandwidth, peak frequency and relative amplitudes for each signal type within an individual. For this purpose, a few individuals of Instar III that produced all four signal types were selected. Note that these were small sample sizes and used for preliminary analyses to identify whether the four signal types differed at all in their vibration characteristics.

How do signal rates differ between instars?

Goal **iv** was to assess how vibratory signals types and rates differ between instars. To achieve this goal, simultaneous laser and video recordings with only established individuals in natural conditions (groups or solitary, without disturbance) were included. Approximately eight hours of video footage with approximately equal time for each instar

were analyzed to calculate signal rates. Excluding any disturbances and keeping the sampling time consistent, signal rates (# of signals/min) were calculated per individual from each instar using last 5 minutes of each recording. Specific questions asked and the respective sampling methods are described in more detail below.

To compare collective signal rates across instars, signal rates (all signal types combined) per individual for each instar, regardless of group size, were calculated. For groups, individual signal rates were calculated using the total number of signals from the group divided by group size and time (i.e. 5 min.) to obtain a signal rate per minute, per individual. For example, if a group comprised 5 individuals that collectively generated 30 signals over 5 min., average signal rate per individual would be calculated as $30/5/5=1.2$ signals/min. To compare differences in signal rates between instar, signal rate data were subjected to Kruskal–Wallis’ test. Significant differences were compared using the Fisher's least significant difference with the package *stats* and *agricolae* ($P < 0.05$). All analyses were performed using the R-software version 3.5.1 (R Core Team 2019).

In addition to comparing collective signal rates across instars I-V, rates for each signal type were also compared. To do so, signal types generated by individuals within an instar were identified using the methods described earlier, and signal rates for each type within an instar were calculated and compared between instars. For groups, individual signal rates for a signal type were calculated using the total number of signals of that particular type from the group divided by group size and time.

How do signal rates differ between solitary individuals across instars?

Signal rates for solitary individuals (i.e. group size=1) from instar I-V were calculated and compared between instars. To compare differences in signal rates between instars, signal rate data were subjected to Kruskal–Wallis’ test. Significant differences were compared using the Fisher's least significant difference with the package *stats* and *agricolae* ($P < 0.05$). All analyses were performed using the R-software version 3.5.1 (R Core Team 2019).

In addition to comparing collective signal rates across solitary individuals of instars I-V, rates for each signal type were also compared. To do so, individual signal rates for each type generated within an instar were calculated and comparisons were made between instars.

How do signal rates differ across group sizes in instars I-III?

Instars I-III were observed to form groups of varying sizes (1-5). We compared signal rates across these group sizes for each instar to understand variation, if any, within an instar with respect to group size. To do so, signal rates of all signals per individual for a particular group size within an instar were calculated, and compared between group sizes. Signal rates were calculated by dividing total signals per group by group size and time.

Rates for each signal type for group sizes within an instar were also calculated to understand signal type variation among group sizes. This was done by calculating individual signal rates for each type by dividing total signals per type per group by group

size and time, and then rates for each signal type for group sizes within an instar were compared.

3.4 Results

3.4.1 Group formation throughout development

The first objective of this study was to test the hypothesis that grouping behaviour changes throughout *D. arcuata* larval development. The first goal tested whether instars differed in their tendency to form groups. Results of the group formation trials show that instars I-III tend to form groups, whereas instars IV and V remained solitary (Figures 3.3, 3.4). Group formation by instars I-III was observed to begin within 1-2 hours from the beginning of trials (Figure 3.3). More than 50% of the individuals from instars I and II had formed groups within 5 h (Instar I=76%; Instar II=56%), and 100% of instar I and 96% of instar II individuals had settled in groups by the end of the 24 h observation period (Figure 3.3). In instar III, 32% of the individuals were in groups by the end of 5 h, however, they dispersed post group-formation resulting in only 8% of the individuals residing in groups by the end of the 24 h period. Also, it took less median time to form groups by instars I,II than instars III, and no groups were formed by instars IV,V (Figure 3.5).

Figure 3.3 Group formation by *D. arcuata* instars over a 24 h observation period. Instars II, III began forming groups at 1 h, instar I at 2 h, whereas instars IV, V remained solitary (represented by bars on negative y-axis) throughout trials. N (total number of trials per instar)=5; n (total number of larvae per trials)=5 (therefore, 25 individuals per instar).

Figure 3.4 Group formation trials for *D. arcuata* instars I-V. Survival curves showing the proportion of solitary larvae over 24 h time period for instars I-V. N (total number of trials per instar)=5; n(total number of larvae per trial)=5 (therefore, 25 individuals per instar)

Figure 3.5 Median time taken by *D. arcuata* instars I-V to form groups. Instars I,II took less median time (<13h) than instar III (>15h) to form groups, and no groups were formed by instars IV and V. ($\chi^2 = 115.04$; d.f.= 4; $P < 0.001$).

The second goal assessed the differences in group size between instars. It is important to note here that as there were only 5 individuals used in each group formation trial, the maximum group size that could have been formed was 5, and therefore not much variation in group sizes can be seen in the current results. Results from group formation trials indicate that instars I-II formed groups of 1-3 individuals, instar III formed groups of 1-2 and instars IV,V remained solitary (i.e. group size=1) throughout the observation period (Figure 3.6). At 2 h, the majority of groups formed by instars I-III were of size 1 (80-95%), changing to most groups of size 2 and 3 at 24 h for instars I, II (90-100%). Instar III formed groups only of size 1 or 2, or both at all the three time points, however, most groups formed were of size=1 at all the three time points (>80%).

Figure 3.6 Variation in group sizes formed by *D. arcuata* instars I-V at 2 h, 5 h, and 24 h. Instar I and II formed group sizes of 1-3, whereas instar III formed groups of 1-2. Instars IV,V remained solitary at each time point (represented by bars of group size=1/solitary on negative y-axis). Dotted horizontal lines represent 0 at y-axis.

Overall, results from the group formation trials support the hypothesis tested in Objective 1 that grouping behaviour changes through *D. arcuata* larval development.

3.4.2 Variation in signal types and rates between larval instars

The second objective of this study assessed how vibratory signalling differs between larval instars. Results for specific goals (**iii** and **iv**) are described below.

What types of signals do D. arcuata produce?

Signal types and mechanisms

Goal **iii** categorized and characterized different vibratory signals produced by *D. arcuata* larvae. Over 6 hours of videos comprising approximately equal footage of all five instars in different group sizes and behavioural contexts confirmed that there are four distinct types of signals, as previously noted by Matheson (2011) (Figure 3.7). These include- buzz scrape (BS), anal scrape (AS), mandible scrape (MS) and mandible drum (MD). Anal scraping is generated by contraction of terminal abdominal segments (A7-A10) while scraping across the leaf surface by a pair of modified posterior setae (PP1) located at the terminal abdominal segment. Buzz scrapes are generated by the tremulation of the caterpillar's body while simultaneously anal scraping. Mandible drumming is generated by striking the opened mandibles on the leaf surface, quickly moving the head up and down. Mandible scraping is generated by scraping the opened mandibles across the leaf surface, by quickly moving the head side to side.

These four signal types were also shown to differ in certain acoustic features (max frequency, duration, relative amplitude) based on comparative analyses of different signal types produced by the same individual within an instar (Table 3.1; Figure 3.7). For example, BS had the longest duration (0.73 ± 0.21 s), closely followed by AS (0.67 ± 0.27 s), whereas both MS and MD had shortest durations of 0.04 ± 0.01 s and 0.03 ± 0 s, respectively. All the signals were observed to be broadband signals, however peak frequencies differed among the signal types, MS had the highest peak frequency at 317.60 ± 128.44 Hz whereas AS showed lowest peak frequency at 21.0 ± 0 Hz (Table 3.1). Further analyses to characterize differences in acoustic features of signal types could be done to understand how they differ between instars, different body sizes, and different distances. However, this was not a goal of the current study.

Analysis of instar specific .wav files also confirmed that 3 of the 4 signals occurred in all instars (AS, MD, MS), but one signal, BS, was absent in instars IV and V. Waveforms and corresponding spectrograms for each signal type generated by *D. arcuata* instars can be seen in Figure 3.7.

We also assessed if there were any changes in one of the morphological features previously identified to be associated with vibratory signalling in *D. arcuata* larvae (i.e. the PP1 seta, or, ‘anal oar’). Comparison of anal oar SEMs revealed that, aside from size differences, there were differences primarily between instar I and the other instars in the shape: in instar I the PP1 is peg-shaped, whereas in instars II-V it is ‘oar’ shaped (Figure 3.8).

Table 3.1 Temporal, spectral characteristics and relative amplitude of the vibratory signals produced by third instar *D. arcuata* caterpillars. N= number of individuals that signals were taken from; n= number of signals analyzed. BS= Buzz Scrape; AS=Anal Scrape; MS=Mandible Scrape; MD=Mandible Drum.

Signal	N	n	Duration ±SD (s)	Bandwidth (-20dB) ±SD (Hz)	Max Frequency ±SD (Hz)	Relative Amplitude ±SD
BS	2	5	0.73±0.21	187.98±39.42	56.00±11.78	89939.00±57758.11
AS	2	5	0.67±0.27	91.86±34.23	21.50±0	78341.60±40220.83
MS	2	4	0.04±0.01	299.45±83.40	317.60±128.44	99343.25±41186.63
MD	2	6	0.03±0	406.23±221.96	96.90±11.83	228341.33±130085.15

Figure 3.7 Types of signals generated by *D. arcuata* larvae. (a) Drawings representing structures and/or body movements associated with each signal type; (b) Waveform and spectrogram showing the four signal types recorded from a solitary instar III in its shelter using a laser vibrometer, (c) Waveform and spectrogram showing the three signal types recorded from a solitary instar V in its shelter using a laser vibrometer. AS- Anal Scrape; MD=Mandible Drum, BS=Buzz Scrape; MS=Mandible Drum.

Figure 3.8 Scanning electron micrographs (SEMs) of anal oars of *D. arcuata* larvae. a) posterior view of terminal abdominal segment (A10) with anal oar (occurring ventral to the anus; anal oars circled in red) from instar I. Scale bar=100 μ m b) close-up of anal oar from same instar I; c) posterior view of terminal abdominal segment (A10) with anal oar from instar II. Scale bar=100 μ m d) close-up of anal oars from instar II. Red circles in images a) and c) highlight the pair of anal oars. No differences in shapes of anal oar were observed from instar II-V, hence SEMs not included here.

Goal **iv** was to assess how vibratory signals rates differ between instars. Results obtained for this goal are described below.

How do signal rates differ between instars?

To compare signalling rates between instars, first the collective signal rates per individual for each instar were calculated regardless of their group size. Here, signal rates represent the combination of all signal types generated by an individual in an instar over a time period of 5 minutes per group. Highest signalling rates were observed in instars I-III, whereas lowest signalling rates were observed in instars IV and V (Table 3.2) (Figure 3.9; Kruskal–Wallis' and Fisher's LSD, $\chi^2 = 23.52$; d.f.= 4; $P < 0.001$)

Also, rates for each signal type per individual were calculated for each instar regardless of their group size. In all the instars, signalling rate was observed to be highest for anal scrape and lowest for mandible scrape among all the signal types generated by individuals within a particular instar (See Table 3.3; Figure 3.10). Also, no buzz scraping was observed in instars IV and V. (Table 3.3; Figure 3.10). It must be noted that the signals analyzed here were recorded from individuals only in their natural conditions (i.e. groups or solitary). Therefore, although the late instars generate a total of three signal types (AS,MS,MD) during territorial encounters with conspecific intruders, AS is generated most often while solitary.

Table 3.2 Mean±SD signal rates (#/min.) per individual generated by *D. arcuata* larvae across instars, regardless of group size. N= total number of individuals studied; n=total number of signals.

Instar	N	n	Signal Rate* ±SD (#/min.)
I	19	497	6.21±4.90 ^a
II	30	922	7.85±8.52 ^a
III	14	712	9.85±7.54 ^a
IV	8	34	0.88±1.50 ^b
V	8	16	0.40±0.77 ^b

*Mean±SD followed by same letter in the column do not differ significantly, as assessed by multiple comparison for contrast test(p=0.05)

Table 3.3 Mean±SD signal rates (#/min.) for each signal type generated by *D. arcuata* instars I-V, regardless of group size. N= total number of individuals studied; n=total number of signals. AS was generated at highest rates and MS at lowest rates, as indicated in bold. BS= Buzz Scrape; AS=Anal Scrape; MD=Mandible Drum; MS=Mandible scrape.

Signal	N	n	Instar I	N	n	Instar II	N	n	Instar III	N	n	Instar IV	N	n	Instar V
BS	19	172	1.55±2.21	30	219	1.48±1.09	14	114	1.43±1.97	8	0	0	8	0	0
AS	19	280	3.94±4.56	30	403	4.36±6.93	14	257	4.24±3.88	8	22	0.88±1.50	8	16	0.40±0.77
MD	19	32	0.64±1.95	30	277	1.93±1.94	14	298	3.74±4.95	8	12	0	8	0	0
MS	19	13	0.08±0.14	30	23	0.09±0.16	14	43	0.44±0.95	8	0	0	8	0	0

Figure 3.9 Signal rates (#/min.) per individual generated by *D. arcuata* larvae across instars I-V, regardless of signal types and group size. Signalling rates were found to significantly higher for instars I-III than instars IV,V. ($\chi^2 = 23.52$; d.f.= 4; $P < 0.001$).

Figure 3.10 Mean signal rates for each signal type generated by *D. arcuata* instars I-V. Highest signalling rates were observed for AS across all the 5 instars. No BS was observed in instars IV and V, hence no bars. BS=Buzz Scrape; AS=Anal Scrape; MD=Mandible Drum; MS=Mandible Scrape.

How do signal rates collectively differ across solitary groups (i.e. group size=1) between instars?

After finding differences in signal rates across instars regardless of the group size they resided in, we wanted to assess if signal rates differ between individuals in solitary shelters (i.e. group size=1) across instars. If vibratory signals are used for recruitment, we might predict higher signalling rates for solitary early instars (I-III) (that tend to group) than late instars (IV,V) (that do not group). Results show that signal rates for instars I-III were significantly higher than instars IV,V (Table 3.4; Figure 3.11) (Kruskal–Wallis', Fisher's LSD, $\chi^2 = 18.16$; d.f.= 4; $P < 0.01$).

Table 3.4 Mean $\pm$ SD signal rates (#/min.) for solitary individuals of *D. arcuata* instars I-V. N= total number of solitary individuals studied.

Instar	N	Signal Rate* (#/min.)
I	6	7.17 $\pm$ 5.54 ^a
II	4	14.65 $\pm$ 12.63 ^a
III	5	7.8 $\pm$ 5.72 ^a
IV	8	0.35 $\pm$ 0.76 ^b
V	8	0.4 $\pm$ 0.77 ^b

*Mean $\pm$ SD followed by same letter in the column do not differ significantly, as assessed by multiple comparison for contrast test(p<0.05).

Figure 3.11 Signal rates per individual generated by solitary (i.e. group size=1) *D. arcuata* larvae across instars I-V. Signalling rates were found to significantly higher for instars I-III than instars IV,V. ($\chi^2 = 18.16$; d.f.= 4; $P < 0.01$).

When comparing signal rates for types across solitary individuals from instars I-V, rates were found to be to highest for anal scrape and lowest for mandible scrape (Table 3.5; Figure 3.12). No buzz scrapes were generated by instars IV,V.

Table 3.5 Mean $\pm$ SD signal rates (#/min.) for each signal type generated by solitary individuals from *D. arcuata* instars I-V. N= total number of solitary individuals studied; n=total number of signals. AS was generated at highest rates and MS at lowest rates, as indicated in bold. BS= Buzz Scrape; AS=Anal Scrape; MD=Mandible Drum; MS=Mandible scrape.

Signal	N	n	Instar I	N	n	Instar II	N	n	Instar III	N	n	Instar IV	N	n	Instar V
BS	5	5	1.17 $\pm$ 2.28	4	4	2.15 $\pm$ 1.17	6	4	0.97 $\pm$ 1.51	8	0	0	8	0	0
AS	5	5	4.43$\pm$5.92	4	4	9.75$\pm$10.52	6	5	5.57$\pm$4.25	8	4	0.63$\pm$0.90	8	2	0.36$\pm$0.73
MD	5	2	1.07 $\pm$ 2.52	4	3	2.75 $\pm$ 3.03	6	4	2.53 $\pm$ 4.00	8	1	0.34 $\pm$ 0.91	8	0	0
MS	5	1	0.03$\pm$0.08	4	0	0	6	1	0.03$\pm$0.08	8	0	0	8	0	0

Figure 3.12 Mean $\pm$ SD signal rates (#/min.) for each signal type generated by solitary individuals from *D. arcuata* instars I-V. BS= Buzz Scrape; AS=Anal Scrape; MD=Mandible Drum; MS=Mandible scrape.

How do signal rates differ across group sizes in instars I-III?

After identifying higher signal rates in solitary individuals of group-forming instars I-III, we then explored if signal rates differed between group sizes within instars I-III. If solitary individuals use vibratory signals for recruitment, then we might predict their signal rates per individual to be higher than for individuals already in groups, and would see a decline in signal rates with increasing group size. This was done for instars I-III as they were observed to form groups of varying sizes (1-5). Individual signal rates (#/min.) for each group size within an instar were calculated. In instar I, the total number of groups for each group size (N) were as follows- Group size 1= 6; size 2=1; size 3=2; size 4=0; size 5=1; N for instar II, Group size 1=4; size 2=3; size 3=3; size 4=1; size 5=2; and N for instar III, Group size 1=6; size 2= 4; size3-5=0. In early instars (I,II) the highest signalling rate was observed for a group size of 1, whereas in instar III the highest signalling rate was observed for a group size of 2 (see Figure 3.13).

How do rates for signal types differ within group sizes?

We then studied how rates of signal types differed between individuals in group of different sizes. If solitary individuals use vibratory signals for recruitment, then identifying particular signals made by smaller groups may help us identify the specific signal types that might be used in recruitment. In early instars (I,II) the highest signalling rate was observed for AS in group size=1 (i.e. solitary), but in instar III the highest signalling rate was observed for MD, in group size=2 (Figure 3.14).

Figure 3.13 Mean $\pm$ SD signal rates (#/min.) for individuals across group sizes of 1-5 within instars I-III. In instars I,II highest signalling rates were observed in group size=1 (i.e. solitary), whereas highest signalling rates were observed for group size=2 in instar III.

Figure 3.14 Mean $\pm$ SD signal rates for each signal type for individuals across group sizes of 1-5 within instars I-III. Highest rates for AS were observed in group size=1 (i.e. solitary) in instars I,II, whereas BS was generated at highest rates in group size=1 (i.e. solitary) in instar III.

3.5 Discussion

Many larval insects live in social groups, and there is evidence that they need to communicate for coordinating group activities (e.g. Hograefe 1984; Seeley 1995; Costa and Pierce 1997; Fitzgerald and Pescador-Rubio 2002; Wirth et al. 2003; Costa et al. 2004; Fletcher 2007; Despland and Santacruz-Endara 2016). However, the mechanisms to form and maintain groups are not very well understood. The current and previous chapter of this thesis took a first step in exploring the possible role that vibrational communication might play in social communication in the masked birch caterpillar, *D. arcuata*, by identifying changes in grouping and signalling behaviours between identified instars. The results showed instar specific changes in both grouping behaviour and signalling behaviour. Documenting this information will allow us to pose and test specific hypotheses concerning how vibratory signalling mediates social grouping in masked birch caterpillars in future studies (see Chapter 4 of this thesis).

Drepana arcuata grouping behaviour changes during larval development

In the current study we found that early instars (I-II) formed groups of various sizes, whereas late instars (IV,V) remained solitary, and instar III was “transitional”. A very interesting finding worth highlighting here is that the tendency to seek conspecifics to form groups by the tiny early instars is quite ‘strong’ as they had to travel a lot farther than other instars (given the differences in their body lengths) to locate nestmates. This leads us to the question- why do they do this? There are numerous examples from other caterpillar species forming groups for different benefits associated with living in groups (e.g. Reader and Hochuli 2003; Costa 2006; Despland 2019). In *D. arcuata* larvae, there are at present no

studies that have experimentally tested “why” they form groups, but they have been observed to have higher survival rates when reared in groups compared to solitary (Chanchal Yadav, personal observation). In fact, higher survival rates in group-living vs. solitary individuals have been observed in other caterpillar species (Stamp 1980; Costa and Pierce 1997; Hunter 2000). Higher survival rates in group living *D. arcuata* could be a result of potential benefits that individuals might derive from living in groups, as seen in other caterpillars species. In *D. arcuata*, for example, we propose that collective feeding and silk shelter construction might be responsible for enhanced growth rates as it may be difficult to cut through leaves/initiate feeding because of small mandibles in early instars (as suggested by Hochuli 2001 in other species). Also collective shelter construction would reduce the time a solitary individual would spend constructing a shelter by itself. Shelters may provide an optimal local microclimate for growth. As noted earlier, the size of groups formed in our group formation trials was restricted by the sample size of five individuals per trial, so groups were small, ranging from 1 to 5 individuals. While it is possible that group sizes are larger in nature, anecdotal observations of wild caught larvae on birch leaves suggest that such group sizes reflect the natural conditions (J. Yack, unpublished). Another important question regarding grouping observed in *D. arcuata* larvae is- how do they do this? As seen in other social larval insects, this might be achieved through different means of communication such as chemical, tactile, or vibroacoustic (Costa 2006, 2010).

All instars produce vibratory signalling

If vibratory signalling is involved in any type of social interaction in *D. arcuata* larvae, then we first need to confirm that instars that form social groups are capable of generating

the signals. Based on the body movements and signal characteristics, we confirmed four types of signals (BS, AS, MD, MS) for *D. arcuata* larvae, that all instars were capable of signalling, but that there were differences in the types and rates of signals produced per instar. Vibratory communication could be used in different contexts as seen in other larval insects, including, but not limited to, food recruitment and grouping (Hoeagraefe 1984; Cocroft 2001; Fletcher 2007, 2008), territoriality (Yack et al. 2001; Fletcher et al. 2006; Scott et al. 2010), predatory detection and avoidance (Castellanos and Barbosa 2006; Gish et al. 2012). However, it was not the purpose of the current study to explore social interactions between individuals, or which individuals signalled, or the context; this should be explored in future study. Our focus was to perform some preliminary analyses to assess how signals may differ between social and solitary instars so that we could identify possible hypotheses to test in subsequent experiments (see Chapter 4).

How does signalling differ between instars?

We found that while instars I-III generate four signal types (BS,AS,MS,MD), one signal type (BS) was absent in late instars (IV,V). Why there is a difference in the types of signals produced by early vs. late instars could relate to differences in function of the signals (e.g. social interactions [recruitment, group coordination, task allocation etc.] vs. territoriality), but also efficacy of signal transmission. These hypotheses would have to be tested in future experiments. For example, social interactions between individuals for forming groups could be examined carefully to identify relationships between signal types and particular activities. If BS, for example, is used for group formation and/or maintenance, we would

expect this signal type to be generated often (i.e. at high rates) during these activities/events.

Our results clearly showed that vibratory signalling rates differ between instars, when individuals are in established shelters on birch leaves, without disturbance from the experimenter or external larvae. For example, early instars generate more signals per individual overall than late instars. There could be different reasons explaining why the signal rates are higher in early instars. Higher signal rates might indicate their use in, for example, recruiting conspecifics/advertising feeding site (e.g. as seen in sawfly larvae [Hograefe 1984; Fletcher 2007]), coordinating activities in groups such as feeding, resting, shelter construction, establishing territories within groups/spacing (as suggested by Matheson 2011) or perhaps in competition between nestmates within a group while feeding. Based on our results, we argue that one possible function of signalling in early instars is for social recruitment. This is based on the following arguments: (a) group forming instars produced more signals than instars that do not form groups; (b) within a particular instar that forms groups, individuals in solitary shelters signalled more per individual than those in groups (in early instars I,II), indicating that these signals are used for recruitment. Moreover, it appears that AS may function in grouping, as this was the most prominent signal generated by individuals and instars most likely to recruit. Collectively, these findings provide some preliminary support that vibrations may be used for group formation/recruitment events. This hypothesis on vibratory recruitment is formally tested in the next chapter (Chapter 4).

Chapter 4: Invitation by vibration: recruitment to feeding shelters by vibrating caterpillars

This chapter is an adaptation of the following publication:

Yadav, C., Guedes, R.N.C., Matheson, S.M., Timbers, T.A., & Yack, J.E. (2017). Invitation by vibration: recruitment to feeding shelters by vibrating caterpillars. *Behavioural Ecology & Sociobiology*. 71, 5.

Statement of contribution:

C. Yadav collected the experimental data, performed data analysis, prepared the figures and wrote the paper; R.N.C. Guedes assisted with some of the statistical analyses relating to signalling and behaviour and choice experiments; S.M. Matheson and T.A. Timbers contributed to data collection; J.E. Yack supervised the project, contributed with experimental design as well as figure preparation and editing the manuscript.

*Note that this paper was published before all of the data for Chapter 3 were collected. Therefore, the reader may note some redundancy in some of the analyses with Chapters 3 and 4.

4.1 Abstract

Sociality is widespread in caterpillars, but the communication mechanisms used for group formation and cohesion are poorly understood. Here, we present the first evidence that caterpillars produce complex vibratory signals to advertise food and shelter sites to conspecifics. We first tested the hypothesis that early instars of the masked birch caterpillar (*Drepana arcuata*) actively form groups. Larvae placed alone on different leaves of a birch twig began assembling within minutes and forming groups of 2–6 at a median time of 2 h. In Y-choice experiments, larvae joined arms occupied by conspecifics significantly more frequently than unoccupied arms. To test the hypothesis that group formation is vibration-mediated, signals were monitored in solitary residents of silk leaf shelters before and during natural recruitment events. Four distinct signal types were recorded: anal scraping, mandible drumming, mandible scraping, and buzz scraping. Anal scraping and buzz scraping were the most common in residents prior to being approached, and these signals were strongly correlated to feeding and laying silk. Signalling occurred in 100% of residents, and higher signal rates resulted in significantly faster recruitment times. As a recruit approached a resident, complex signalling interactions occurred, which may communicate information about resource quality or location. We conclude that caterpillars, similar to other social animals, use acoustic communication to advertise resources. The vibratory signalling repertoire of these tiny caterpillars exhibits a complexity rivaling that of eusocial insects. Further investigations of vibroacoustic communication are essential to fully appreciate the intricacies of social interactions in caterpillars and other juvenile insects.

4.2 Significance statement

Group living provides many survival benefits to juvenile insects such as caterpillars, but little is known about the communication signals mediating social interactions such as group formation. Our study shows that caterpillars use vibration signals to “invite” conspecifics to social gatherings. Pinhead sized early-instar caterpillars (*Drepana arcuata*) are capable of locating conspecifics on birch leaves to form small groups. But how do they accomplish this? We report that individual resident caterpillars established in a silk shelter produced complex vibrations by dragging their anal segments, scraping and drumming their mouthparts, and tremulating their bodies to advertise a feeding spot and shelter. These results provide the first evidence that caterpillars use vibratory signalling to form social groups, providing insight into the poorly understood role of vibratory communication in juvenile insects.

4.3 Introduction

“Communication is the glue that binds individuals to one another” (Costa 2006). Group living is widespread throughout the class Insecta, occurring in more than 12 orders, and communication is essential for mediating the formation, cohesion, and organization of such groups (Costa 2006). While communication signals and mechanisms have been extensively studied in the eusocial insects such as bees, wasps, ants, and termites, much less is understood of these mechanisms in the other social insects, such as caterpillars (Costa 2006; Cocroft and Hamel 2010).

Many species of larval Lepidoptera across a diverse array of taxa form social groups at some stage of their development (Costa and Pierce 1997; Zalucki et al. 2002; Costa 2006). Groups can range in size, from two to several hundreds of individuals, and complexity, from patch-restricted foragers that share a feeding shelter to large groups that exhibit coordinated foraging expeditions and division of labor (Fitzgerald and Peterson 1988; Costa 2006; Dussutour et al. 2008). Benefits derived from group living in caterpillars include predator defense, feeding facilitation, and thermoregulation (Costa and Pierce 1997; Prokopy and Roitberg 2001; Costa 2006). While such benefits have been well documented, the communication mechanisms necessary for mediating social interactions, such as group formation and coordination of group activities, are poorly understood (Costa and Pierce 1997; Costa 2006). Group formation by neonates may be initially facilitated by adult females, as many species lay eggs in rows or clusters or multiple females may lay on the same host plant, ensuring that larvae are in close proximity to one another (Prokopy and Roitberg 2001). However, female egg-laying habits are usually not sufficient for larvae to form and maintain social groups that must respond to changing food and environmental conditions (Fitzgerald and Peterson 1988; Costa and Pierce 1997; Zalucki et al. 2002). Most reported examples of social communication in caterpillars involve chemical markers, often as pheromone trails laid by processionary species, and in some species, tactile contact is used for maintaining processions (Costa and Pierce 1997). There is little evidence that visual communication signals are used (Costa and Pierce 1997; Prokopy and Roitberg 2001), probably owing to the simple eyes that larval insects possess (Warrant et al. 2003). Vibratory communication should be optimal for short-range communication in small plant borne insects (Virant-Doberlet and Cokl 2004; Cocroft and Rodriguez 2005; Yack 2016),

but at present, there is no direct evidence for vibratory-mediated group coordination in caterpillars. To expand our knowledge of the functions and mechanisms of social interactions in caterpillars, detailed observations of the insects on their host plants during social activities, in addition to experimental manipulations, are essential. This study is the first to explore the role of vibratory communication as a mechanism of social recruitment in caterpillars.

Early-instar larvae of the masked birch caterpillar, *Drepana arcuata* (Lepidoptera: Drepanidae), are good models to test hypotheses on vibratory-mediated group formation. The species occurs throughout northeastern North America (Rose and Lindquist 1997) and exhibits five larval instars that feed on birch (*Betula* spp.) and alder leaves (*Alnus* spp.) (Figure 4.1). Late instars (third to fifth) live solitarily on individual leaves and use vibratory signals during territorial disputes over silk shelters (Yack et al. 2001; Scott et al. 2010; Guedes et al. 2012; Yack et al. 2014). Early instars (first and second) on the other hand have been observed to form small groups (Yack et al. 2001). Moreover, early instars have been noted to generate vibratory signals (Yack et al. 2001), although these signals have not been formally studied. Finally, because these insects reside in open leaf shelters, their behaviours and vibratory signals are amenable to being simultaneously monitored during social interactions with minimal disruption.

Figure 4.1 Eggs and early-instar larvae of *D. arcuata*. a Birch leaf (*Betula papyrifera*) showing a row of 13 egg cases (inside oval) and 2 groups of early-instar larvae (indicated by arrows) comprising 15 individuals in total. Scale bar 5 mm. b Row of unhatched eggs. Scale bar 3 mm. c Group of five first-instar larvae inside a silk shelter. Scale bar 2 mm.

We first tested the hypothesis that early instars actively form groups. We predicted that (1) larvae placed on separate leaves of a birch twig will form groups; (2) larvae will not always establish shelters at the same location of a leaf; and (3) in Y-choice experiments, larvae will choose to join conspecifics over choices with no larvae present. Second, we tested the hypothesis that vibratory signals are associated with group formation. We predicted that (1) early instars generate vibratory signals; (2) solitary larvae established in shelters (residents) will signal; (3) vibratory signals generated by residents are associated with activities that would be attractive to potential recruits, such as feeding and shelter building; (4) vibratory signals are associated with recruitment events; (5) residents signal more than potential recruits; and (6) residents that signal at higher rates recruit conspecifics more quickly. Our results show that during group formation events, caterpillars engage in complex vibratory signalling patterns that are unprecedented for most social insects.

4.4 Methods

4.4.1 Study animals

Female *D. arcuata* (Lepidoptera: Drepanidae) moths were collected at ultraviolet lights at the Queen's University Biology Station (Chaffey's Lock, Ontario, Canada, 44.5788° N, 76.3195° W) and other regions near Ottawa, ON, Canada (45.4215° N, 75.6972° W) between May and September 2010–2015. Moths oviposited on cuttings of paper birch (*D. arcuata*) held in water-filled plastic vials and on paper bag clippings. Hatchlings were carefully transferred to fresh birch cuttings with a fine paintbrush and reared indoors (18–24 °C and 16L:18D) in glass jars (~22 cm long, 8 cm wide). Only the early instars (first and second) were used in experiments. Individuals used in experiments were of mixed

parentage (i.e., larvae were not necessarily kin) as eggs from multiple females were mixed. Due to the nature of the experiments, which involved direct measurement of signals from focal individuals, blind scoring was not possible.

4.4.2 *Group formation experiment*

The first hypothesis predicts that larvae placed on separate leaves of a birch twig will move from their respective individual leaves to form groups. To test this, we conducted 30 “group formation” experiments. In each experiment, a birch twig (12–15 cm long) with five or six leaves (each 7–9 cm long and 4–6 cm wide) was positioned in a water-filled plastic vial (Figure 4.2a). Using a fine paintbrush, one larva was placed on each of the five leaves within a 1-min period from placement of the first larva. Larvae were left undisturbed in a plastic bin and monitored for the number and sizes of groups at the following time points: 0, 0.5, 1, 2, 3, 4, 5, and 18–24 h. All trials were performed in a greenhouse setting at a temperature of 22–26 °C during daylight hours (10:00–16:00), and no individuals were used more than once. We calculated the percentage of individuals in groups at each time interval over the 30 experiments. To follow the progression of group formation over time, a time-failure analysis (i.e., survival analysis) was performed using Kaplan-Meier estimators (PROC LIFETEST; SAS software, SAS Institute, Cary, NC, USA). Regression analysis was subsequently used to assess the prevalence of group size at the end of group formation, when the groups became stabilized, and its relationship to the number of groups formed (PROC GLM; SAS software, SAS Institute, Cary, NC, USA). We also predicted that larvae would not always form shelters at the same location on the leaf. We noted the locations of shelters at 48 h and analyzed the probability of shelter formation on any given portion of the leaf. This was done by dividing the leaf into four sections (tip, base, outer

edge, and middle; Figure 4.2b), calculating the percentage of shelters in each section, and performing a χ^2 test to determine if shelters in any one region occurred more than expected by chance (i.e., 25%).

Figure 4.2 Group formation experiment. a. Experimental setup where 5–6 early-instar larvae were placed individually on separate leaves of a birch (*Betula papyrifera*) twig. Black arrow points to close up of a leaf showing placement of a larva. b. Areas of leaf where larvae resided in leaf shelters at the end of the experiment. Shelters occurred on all parts of the leaf, but with a preference for the outer edges. c. Percentage of larvae that formed groups or remained solitary during the first 5 h of the observation period. It took a median time of 2 h for the larvae to form groups regardless of the group size as shown by time-failure analysis ($\chi^2 = 21.20$, $p < 0.001$). d. Group sizes ranged from two to six, with prevalence of small group sizes (two larvae per group), negatively related to the number of groups formed.

4.4.3 *Y-choice experiments*

If early-instar larvae actively form groups, we predicted that they would preferentially choose to join conspecifics in a Y-choice experiment. Two different experiments were performed (Figure 4.3). The first assessed whether a test larva would choose the arm of a Y occupied by conspecific larvae over an unoccupied arm. Fresh birch leaves were cut into a Y (3-cm-long, 2.5- mm-wide arms). The Y was clamped into a horizontal position using a paper clip at the base of the test arm and supported by reusable adhesive putty (Staples®). Three larvae were placed on one arm and the other arm was left unoccupied. A fourth larva (the test larva) was then placed within 2–5 s at the base of the Y. The test larva was considered to have “chosen” an arm when it reached the middle of either arm within 1 min of the beginning of the trial. Between trials, positions of the unoccupied and occupied arms were alternated to preclude any positional bias. The second Y-choice experiment assessed whether larvae would be attracted to an arm that had a potential chemical residue of previous occupants. The Y was cut and positioned as described above with the following modifications: Two custom-made lightweight foam barriers (1.5 cm long, 0.7 cm wide) with slits were placed at the base of each arm, and then three larvae were placed on one of the arms distal to the barrier. Larvae were allowed to walk over the arm and were continuously monitored so that they could be placed back on the arm if they attempted to wander over the barrier. The other arm was left unoccupied. After 30 min, the larvae and barrier were removed and the test larva placed at the base of the Y, and its choice of arm recorded using the criterion outlined above. Thirty trials were completed for each experiment, using different larvae and a new Y made from a fresh leaf for each individual

trial. All trials were performed at room temperature (21–24 °C). Statistical analysis was performed using two tailed χ^2 test with GraphPad Prism software.

Figure 4.3 Y-choice experiments testing preferences of test larvae for joining conspecifics. a Bar graph showing that 86.7% of test larvae chose the arm occupied by conspecifics, indicating a significant preference (indicated with an asterisk) for the Yarm occupied by conspecifics ($\chi^2 = 16.1$, $p < 0.0001$). b Bar graph showing that 43.3% of test larvae chose the previously occupied Yarm, indicating no significant preference over a previously unoccupied arm ($\chi^2 = 0.53$, $p < 0.465$).

4.4.4 *Vibration signal types and rates*

Our first goals were to document if and how early instars produced vibration signals, measure signal types and rates for solitary shelter residents, and then determine how signalling is associated with other non-signalling behavioural activities.

Vibration signal types: To identify the diversity of signal types produced by early instars, we reviewed video recordings of larvae that had settled in groups of 1–5 individuals. Larvae were placed randomly on birch twigs containing 3–5 leaves and left undisturbed over a 12-h period during which time they became established in shelters of different group sizes. Vibrations were recorded using a laser-Doppler vibrometer (PVD-100, Polytec Inc., Irvine, CA, USA) (velocity 20 mm/s; high-pass filter off; low-pass filter 20 kHz) by focusing the laser beam on a circle of reflective tape (2 mm diameter) attached to the upper leaf surface within 1–2 cm from a shelter. Trials were simultaneously videotaped using a camcorder (Sony hd-rxr520v Handycam, Sony Corp., Tokyo, Japan) with the output from the laser connected to the microphone port. Vibrations were also recorded as .wav files to a data recorder (Marantz PMD 671, Marantz Corp., Kanagawa, Japan) at a sampling rate of 48 kHz. Trials were performed in an acoustic chamber (C-14A MR, Eckel Industries Ltd., Cambridge, MA, USA). In total, 38 half-hour videos were examined, comprising nine replicates each for groups of two and four individuals and ten replicates each with solitary individuals and groups of five individuals.

Vibration signals of solitary residents: If vibration signals function as recruitment signals, we predicted that solitary individuals established in a shelter would generate

signals and that signalling would be associated with activities attractive to potential recruits, such as feeding or shelter construction. We recorded ten solitary residents using the video and laser recording methods described above. The individual was left undisturbed in its shelter for 5–10 min prior to recording. Time-sequenced files were created using JWatcher Video 1.0 (Blumstein et al. 2010). Video recordings of each trial were watched at reduced speed, and each signal event was time marked over the 30-min period to calculate overall signal rates (i.e., rates of the different signals pooled) as well as rates of each individual signal type. The signal rate data were transformed to $\log_{10}(x + 1)$ and subjected to ANOVA and Fisher's LSD test (PROC GLM; SAS software, SAS Institute, Cary, NC, USA) to compare rates of different signal types. To assess if and how signals were associated with different behavioural states, rates of each signal type were calculated over the total time course of the four most common behaviours, feeding, laying silk, walking, and resting, using JWatcher Video 1.0. A multivariate analysis of variance (MANOVA) and canonical variate analysis (CVA) of rates of each signal type were performed to test if signals would differ among the non-signalling behavioural states. Such results were subsequently subjected to complementary analyses of variance as well as Fisher's LSD test (at $p = 0.05$) (PROC GLM; SAS software, SAS Institute, Cary, NC, USA).

4.4.5 Vibratory signalling during group formation

Another set of experiments was conducted to determine how vibratory signalling was associated with group formation events. A fresh leaf (~10 cm long, ~6 cm wide) was set up for laser and video recordings as described above with the following modifications:

Prior to placing larvae on a leaf, the reflective tape was attached to the upper surface of the leaf 1–2 cm from the leaf base. Five to eight larvae were then placed at random positions on the leaf and left undisturbed for 2.5–3 h or until at least one established group was formed. Fourteen experimental trials were conducted (involving 102 larvae in total). No larvae were reused between trials.

Video and laser recordings were scored for the time it took a resident to establish a shelter or feeding site on the leaf and the time to its first visitor or joiner (see below for definitions). The number and types of vibratory signals generated by caterpillars surrounding recruitment events were also recorded. The following definitions and scoring criteria were established: A resident (hereafter referred to as “R”) is an individual that establishes itself on the leaf and is eventually visited or joined by another caterpillar. By “establishment,” we refer to shelter construction and/or commencement of feeding. A shelter consists of a silk mat on the leaf surface and one or more strands of silk joining edges of the leaf. A visitor (hereafter referred to as “V”) is an individual that comes within 0.5 cm of the R but then leaves, and a joiner (hereafter referred to as “J”) enters the shelter or feeding area and feeds and/or contributes to the shelter construction. A group was considered to be established when the R and J remained together for 30 min or longer. To score signalling surrounding recruitment events, we identified the time frame as the time R was established until 30 s after it was joined or visited. Signalling was scored as a conspecific approached the R through different zones. To do this, the leaf was divided into four 1-cm zones (D–A), with D being the farthest and A being the closest to the resident. A transparent acetate sheet was positioned over the computer monitor screen, and zones scaled based on leaf

dimensions. The scoring session was initiated when the V or J entered zone D (farthest) and was ended 30 s after the V or J entered zone A (closest). The following measurements were obtained: the number and types of signals produced by the R as a V or J traversed through each zone, the number and types of signals produced by the V or J as it passed through each zone, and the time spent by V or J in each zone.

Data from this experiment were analyzed to address predictions 4–6 outlined in the introduction: Prediction 4 stated that vibratory signals are associated with recruitment events. To test this, we asked the following questions about the R signals: (1) Does an R signal prior to being approached by a V or J? This was addressed by noting the % of trials that resulted in R signalling at least once while being approached by V or J through any of the zones; (2) How do signal rates change as V or J approach? Changes in R overall signal rates (regardless of signal type) were measured as V or J approached across the four zones and compared using ANOVA and Tukey's HSD test (at $p = 0.05$; PROC GLM; SAS software, SAS Institute, Cary, NC, USA); and (3) How do the types of R signals change as a V or J approaches? This was analyzed by comparing the proportion of signal types across zones using χ^2 contingency table (4 x 4; $p = 0.05$; PROC FREQ; SAS software, SAS Institute, Cary, NC, USA). We also asked questions about V and J signalling: (4) Do V or J signal? This was reported as the percentage of V + J (i.e., V and J combined) that signal at least once during the trials; (5) Do V + J signal rates change as they approach R? Rates were calculated as for the R signalling and results also analyzed using ANOVA and Tukey's HSD test (at $p = 0.05$); and (6) Do the types of V + J signals differ between zones as they approach the resident? This was analyzed by comparing the proportion of signal

types per zone using a χ^2 contingency table (4×4 ; $p = 0.05$). Prediction 5 stated that R would signal more than V or J. To address this, signal rates of R and V + J were subjected to ANOVA and Tukey's HSD test ($p < 0.05$; transformed to $\log_{10}(x + 1)$; PROC GLM; SAS software, SAS Institute, Cary, NC, USA). We also compared the signal types and proportions for R and J + V using a χ^2 contingency table (4×4 ; $p = 0.05$; PROC FREQ; SAS software, SAS Institute, Cary, NC, USA). Prediction 6 stated that higher R signal rates will result in faster V + J recruitment. To test this, we measured the total number of signals generated by R from the time it started feeding and/or laying silk until a V or J reached within 0.5 cm. A regression analysis was conducted by plotting R signal rates against the time taken by V + J to reach R (PROC GLM; SAS software, SAS Institute, Cary, NC, USA). Finally, we examined if there were differences between signalling rates of J and V, regardless of their distance to the resident using ANOVA (transformed to $\log_{10}(x + 1)$; PROC GLM; SAS software, SAS Institute, Cary, NC, USA).

4.5 Results

4.5.1 Group formation and locations

During group formation experiments, larvae began to establish groups within the first 30 min. The proportion of the population residing in a group steadily increased over time (at 1 h, 48%; 2 h, 62%; 3 h, 70%; 4 h, 76%; 5 h, 84%; and 24 h, 86%; $N = 30$ trials with 172 individuals). The median time taken for group formation was 2 h and stable group sizes were reached within 5 h (Figure 4.2c). Group sizes ranged from two to six and were negatively related to the numbers of groups formed, with a high number of smaller (i.e., less than four larvae) groups (Figure 4.2d). Larvae do not always form shelters on the same

region of the leaf (Figure 4.2b), but the probability of forming a shelter on any of the four regions is different than expected by chance ($p < 0.01$), with a higher prevalence of shelters occurring at the outer edges.

Figure 4.4 Vibration signals. **a–d** Early-instar (I, II) *Drepana arcuata* produce four distinct types of vibrations using different body parts. **e** Laser vibrometer recording of a solitary resident in its shelter. The first part of the recording shows anal scraping (AS) while feeding; the second part shows the other three signal types (MD, MS, and BS) occurring as the resident is approached. **f–g** Waveforms and spectrograms showing the four signal types.

4.5.2 *Y choice experiments*

In the first Y-choice experiment (occupied vs. unoccupied), 26 of 30 test larvae chose the arm with larvae over the arm without larvae, showing a significant preference for occupied arms ($\chi^2 = 16.1$, $p < 0.0001$) (Figure 4.3a). In the second experiment (previously occupied vs. unoccupied), 13 of 30 test larvae chose the previously occupied arm, not exhibiting a preference ($\chi^2 = 0.53$, $p < 0.47$) (Figure 4.3b). These results indicate that early-instar larvae are attracted to conspecifics and not to putative chemical residues on the leaves.

4.5.3 *Vibration signal types*

Four distinct signal types were identified: anal scraping, mandible drumming, buzz scraping, and mandible scraping (Figure 4.4; Appendix Figure A4.1; Appendix Files A4.1-4.3). Anal scrapes (hereafter referred to as AS) are produced when the caterpillar contracts its terminal abdominal segments (A7–A10) anteriorly and scrapes a pair of thickened posterior proctor setae (PP1) located on the terminal abdominal segment across the leaf surface (Figure 4.4a). Mandible drums (hereafter referred to as MD) are produced by vertically striking the leaf surface with opened mandibles (Figure 4.4b). A buzz scrape (hereafter referred to as BS) is produced when the caterpillar tremulates its body while anal scraping (Figure 4.4c). Tremulation is a fast oscillation of the body, transmitting vibrations to the plant substrate (Hill 2008; Yack 2016). Mandible scrapes (hereafter referred to as MS) are produced by rapidly scraping opened mandibles laterally against the leaf surface (Figure 4.4d).

4.5.4 *Signalling rates and associated behavioural states of residents*

Undisturbed residents generate 5.57 ± 3.69 signals/min (all signals combined; $N = 10$ individuals $\times$ 30 min each). There were significant differences in the rates of different signal types ($F_{3,36} = 9.94$, $p < 0.001$; ANOVA and Fisher's LSD test at $p = 0.05$) (Figure 4.5). Anal scrapes occurred at significantly higher rates than all other signal types, at 3.50 ± 0.90 signals/min, followed by BS (1.42 ± 0.45), MD(0.56 ± 0.29), and MS (0.087 ± 0.051) (Figure 4.5).

Figure 4.5 Vibration signal types and rates for solitary early-instar *D. arcuata* larvae in shelters. Rates for each signal type are significantly different from each other (ANOVA and Fisher's LSD, $F_{3,36} = 9.94$, $p < 0.001$). Box plots indicate the range of data dispersion (lower and upper quartiles and extreme values), mean (dashed line), median (solid line), and outliers (symbols).

Signalling occurred during all four behavioural states—eating, laying silk, walking, and resting—although the types and rates of signals differed between these activities (Table 4.1; Figure 4.6). Early-instar larvae feed by chewing on the leaf surface (i.e., skeletonization) and, while chewing, generate AS regularly at a rate of $11.5 \pm 7.55/\text{min}$ (Figure 4.4e; Appendix File A4.2). While constructing the silk shelter, AS was the most frequently occurring signal ($2.66 \pm 4.08/\text{min}$) (Appendix File A4.2), with a small amount of BS, and no MD or MS. While adding silk to the shelter, the R's anal segment is in contact with the leaf and AS is performed while the upper part of the body is on the silk shelter (Appendix File A4.2), whereas, in order to BS, the caterpillar briefly descends from the silk shelter, performs BS, and then resumes silk-laying activity. While walking, BS was the most common signal (6.5 ± 3.8 signals/min), with small amounts of the other three signal types. Caterpillars generated BS while walking within the shelter from one activity to the next. While resting, the most common signal was BS (1.68 ± 3.79 signals/min), with smaller amounts of the other three signal types (Figure 4.6).

Table 4.1 Vibratory signals in early-instar *D. arcuata* caterpillars during different behavioural states. Signal rates (#/min) $\pm$ SD generated during four behavioural states—resting, walking, eating, and laying silk.

	Buzz scrape	Mandible Scrape	Anal Scrape	Mandible Drum
Resting	1.68 $\pm$ 3.79	0.09 $\pm$ 0.38	0.08 $\pm$ 0.29	0.77 $\pm$ 2.33
Walking	6.5 $\pm$ 3.83	0.29 $\pm$ 0.97	0.36 $\pm$ 0.87	1 $\pm$ 1.99
Eating	0	0	11.48 $\pm$ 7.55	0
Laying Silk	0.29 $\pm$ 1.39	0	2.66 $\pm$ 4.08	0

Figure 4.6 Relationships between behavioural states (i.e., non-signalling activities) and vibratory signals in early-instar *D. arcuata* larvae. a. Ordination diagram of the relationship among behaviours based on the rate of vibratory signals; vibration signal rate profiles during walking and resting are indistinguishable from each other but different from eating and laying silk, which are different from each other. b., c. Rates of anal scrape and buzz scrape signals, respectively, during the four non-signalling behavioural states. Anal scrape and buzz scrape differ significantly during four different behaviours ($F_{3,30} > 12.95$, $p < 0.05$), with anal scrape occurring significantly more during eating, whereas buzz scrape occurring more during walking. Box plots (b, c) indicate the range of data dispersion (lower and upper quartiles and extreme values), mean (dashed line), median (solid line), and outliers (symbols).

We also asked if the rates of each signal type—AS, BS, MD, and MS—differed across the four behaviour states. MANOVA indicated overall significant differences in signal rates across behavioural states (Wilks' lambda = 0.0713, $F_{\text{appr.}} = 9.48$, $df_{\text{num;den}} = 12;66$, $p < 0.001$). A CVA indicated that the signalling rate patterns of larva while either walking or resting are indistinguishable but differ from those when the larva is eating and laying silk, which also differ from each other (Table 4.2; Figure 4.6a). Such differences were mainly due to AS and BS, which were the main contributors for the composition of the two significant CVA axes (i.e., provided higher canonical loads; Table 4.2); AS and BS were significantly different across each of the four behavioural states ($F_{3,30} > 12.95$, $p < 0.05$) with AS prevailing while the larvae are eating and BS prevailing when the larvae are walking (Figures 4.6b, c).

Table 4.2 Canonical loadings from canonical variate analysis (CVA) of the signal rates of early-instar *D. arcuata* associated with four behavioural states (eating, laying silk, resting, and walking) (MANOVA: Wilks' lambda = 0.0713, $F_{\text{appr.}} = 9.48$, $df_{\text{num;den}} = 12;66$, $p < 0.001$).

Signals	Canonical axes	
	1 st	2 nd
Anal scrape ^a	0.76	0.61
Buzz scrape ^b	-0.42	0.51
Mandible drum	-0.14	-0.15
Mandible scrape	-0.13	0.19
Proportion of variance explained	0.81	0.18
$F_{\text{appr.}}$	9.48	4.27
Degrees of freedom (num; den)	12; 66	6; 52
P	< 0.001	0.001

^{a,b}Anal scraping and buzz scraping were the main contributors to the two significant CVA axes, highlighted in bold

4.5.5 *Vibrational signalling associated with group formation*

Of the 14 group formation experiments, four were excluded owing to poor quality of laser recordings due to changes in the leaf position over the course of the experiment. Of the 10 remaining experiments, data for the first 20 established R that were joined or visited were analyzed. For these 20 R, it took 41.17 ± 38.02 min from the beginning of the trial to become established on the leaf. Of these 20 R, 10 were joined and 10 were visited within the observation period. Mean times for V and J to arrive at R's shelter were 6 ± 7.04 min (median time of 3 min) and 25 ± 20.65 min (median time of 24 min), respectively.

Resident signalling during recruitment events: All 20 residents (100%) generated vibratory signals prior to being joined or visited (Figure 4.4; Appendix File A4.3). As the potential recruit approached from zone D (farthest from resident) to A (closest to resident), R signal rates increased significantly (ANOVA and Tukey's HSD test; $F_{3,76} = 6.89$, $p < 0.001$) (Figure 4.7a). The proportion of signal types produced by the R also changed significantly as it was approached (χ^2 contingency test; $\chi^2 = 62.55$, $df = 9$, $p < 0.001$) with AS prevailing at farther distances (zone D) and BS prevailing at closer distances (zone A) (Figure 4.7a).

Figure 4.7 Vibratory signalling during grouping events in early-instar *D. arcuata*. a Vibration signal rates of the resident (R) as it is approached by a potential recruit (ultimate visitor (V) or joiner (J)) as the latter passes through zones D to A (far to near the resident). Signal rates and proportion of signal types are significantly different across zones (ANOVA and Tukey's test, $F_{3,76} = 6.89$, $p < 0.001$; $\chi^2 = 62.55$, $df = 9$, $p < 0.001$). The leaf inset is a schematic representation of distance zones D to A with the R shown at the leaf tip. However, in trials, R may have been at any location on the leaf. b Vibration signal rates of the potential recruit as the latter approaches the resident. Signal rates vary significantly across zones (ANOVA and Tukey's test, $F_{3,72} = 3.50$, $p < 0.02$); however, there is no significant difference in the proportion of signal types across zones D to A ($\chi^2 = 8.71$, $df = 6$, $p < 0.19$).

Visitor and joiner signalling during recruitment events: Recruits also signalled, but at lower rates and with different signal types than R (Figure 4.7b). V + J signalled at least once in 60% of the 20 events, and signal rates varied significantly between zones (ANOVA and Tukey's HSD test; $F_{3,72} = 3.50$, $p = 0.02$), although much less so than observed in R signals (Figure 4.7a, b). However, the proportion of signal types did not vary significantly from zones D to A ($\chi^2 = 4.07$, $df = 9$, $p = 0.91$) with BS prevailing throughout (Figure 4.7b). Signalling rates were higher overall for residents (7.10 ± 1.40 signals/min) than V + J (2.51 ± 0.62 signals/min) ($F_{1,152} = 14.99$, $p < 0.001$), and this difference was even more pronounced at closer distances ($F_{3,152} = 7.19$, $p < 0.001$); significant differences prevail for each signal type and proportion, which vary with distance between R and recruits (V + J) ($\chi^2 = 85.47$, $df = 9$, $p < 0.001$) (Figure 4.7a, b). Signal rates of J (3.22 ± 1.23 signals/min) were higher than those of V (1.57 ± 0.63 signals/min) ($F_{1,72} = 5.36$, $p = 0.02$). There was a significant negative relationship between the R's signal rate and the time taken by the V and J to reach within 0.5 cm of R ($R^2 = 0.35$, $F_{1,10} = 5.33$, $p = 0.04$) (Figure 4.8).

Figure 4.8 Vibration signal rates relative to the duration and success of a recruitment event. Residents with overall higher signal rates recruit conspecifics faster than those with lower rates ($R^2 = 0.35$, $F_{1,10} = 5.33$, $p = 0.04$)

4.6 Discussion

“...the largest gap in our knowledge of social Lepidoptera lies in the feature most essential to their sociality: communication.” (Costa and Pierce 1997) Social interactions in caterpillars, like for many of the “other social insects,” can be complex, but a full understanding of this complexity hinges upon our understanding of their communication systems (Costa and Pierce 1997; Coccoft and Hamel 2010; Costa 2006). To the best of our knowledge, there has been no evidence to date that vibrations play a role in coordinating social activities between conspecifics in larval Lepidoptera. Considering that there are several reports of larval Lepidoptera using vibrations for territoriality and mutualistic interactions with ants (see Travassos and Pierce 2000; Costa 2006; Scott et al. 2010; Yack 2016), the lack of evidence for vibratory-mediated sociality is surprising. This study provides the first evidence that caterpillars use vibrations to coordinate social activities and, specifically, to advertise food and shelter sites to conspecifics.

Early-instar caterpillars actively form groups

Our results support the hypothesis that early-instar *D. arcuata* actively form groups. During group formation experiments, larvae began assembling in shelters within the first 30 min, and by 5 h, 84% had formed groups. These tiny caterpillars (1–2 mm in length) travelled long distances (up to an estimated 420x their body lengths) before settling in a group. Also, while larvae prefer to form shelters on the edges of a leaf, most likely because leaf edges are more easily drawn together with silk, there was not one consistent location chosen, ruling out the likelihood that larvae simply aggregate at the same location based on physical cues. In Y-choice trials, test larvae chose to join an arm of a birch leaf with

conspecifics over an unoccupied arm. Collectively, these results support the hypothesis that larvae actively seek out conspecifics to form groups. Adult female *D. arcuata* and congeners lay eggs in rows at various locations of the tree, including the upper and lower surfaces of leaves, petioles, and on twigs and branches, and, upon hatching, wander away from the egg cases before becoming established on a leaf (Bryner 1999; unpublished data JEY). Despite where eggs are laid, most neonate Lepidoptera larvae wander in search of a location to settle (Zalucki et al. 2002) and groups form and dissolve as food resources are depleted or as they are confronted with changing environmental conditions. To establish social groups, communication mechanisms are required. We propose that for *D. arcuata*, vibratory communication signals play an important role in this process.

Vibrations advertise food and shelter sites

Our results indicate that vibratory signals function to advertise food and shelter sites to conspecifics, resulting in the formation of small social groups. Acoustic signals are employed by many vertebrates to attract conspecifics to food sources (Bradbury and Vehrencamp 2011), but comparatively little is known about how insects use sounds or vibrations in this context. Vibratory recruitment to a food source is best known for the eusocial insects (e.g., dances of honeybees) (Hunt and Richard 2013), but in the “other social insects,” examples are limited to a few species of nomadic sawfly larvae and treehopper nymphs (Cocroft and Hamel 2010). Until now, there have been no reported examples of vibratory recruitment signals to food sources in caterpillars. Moreover, the vibratory communication system in *D. arcuata*, with four distinct signal types and changing signal dynamics during recruitment, rivals the complexity of food advertisement

signals reported for eusocial insects (cf. Hunt and Richard 2013). To gain insight into this novel form of communication in caterpillars, we discuss our results in the context of other acoustic food advertisement signals in both vertebrates and invertebrates.

Several lines of evidence support the hypothesis that vibrations function to advertise food and shelter resources to conspecifics in early-instar *D. arcuata* : First, solitary Rs always generate signals prior to being approached; second, the R signals significantly more than does the recruit; third, the most frequent signals, AS and BS, are strongly associated with feeding and laying silk; and fourth, higher R signal rates resulted in faster recruitment times. Similar results are reported for advertisement calls in primates (e.g. Caine et al. 1995; Di Bitetti 2003, 2005; Gros-Louis 2004; Slocombe et al. 2010) and birds (e.g. Elgar 1986; Mahurin and Freeberg 2009; Suzuki 2012), where individuals that first locate a resource call to potential recruits and higher call rates result in more successful recruitment. In the eusocial insects, vibratory recruitment signals to food sources are mostly reported for central-place foragers, where a scout advertises a remote food source by generating vibrations (see Hunt and Richard 2013; Hrnčíř and Barth 2014). In the other insect societies (i.e., the non-eusocial insect societies), vibratory-mediated recruitment to food sources has been reported only for a few species of treehopper nymphs and sawfly larvae. In the treehopper *Calloconophora pinguis*, a nymph that locates a food source generates vibrations to recruit conspecifics to the feeding site and higher quality of food is communicated with higher signal rates (Cocroft 2005). In one species of sawfly, *Hemichroa crocea*, larvae produce vibrational signalling while feeding to attract conspecifics to a feeding site (Hograefe 1984). Similar to our observations in *D. arcuata* ,

sawfly larvae “scratch” their terminal abdominal segment on the leaf surface while feeding, and, as for the abovementioned treehopper nymphs, signal rates are proposed to relate to leaf quality. Based on these comparisons to previous studies, we propose that *D. arcuata* residents signal to advertise food and shelter resources to potential recruits and that variation in signal rates functions to advertise the quality of the shelter or food resource. Future studies should investigate the relationship between site quality, signal rates, and recruitment times by manipulating leaf conditions and conducting playback experiments. Our results also showed variation in signalling patterns as R was approached by a potential recruit: First, R signal rates increased as a recruit approached the shelter; second, R signal types changed as the recruit came closer; and third, potential recruits also signalled, but at much lower rates than R and with different signal types. We have not been able to find examples of similar signalling patterns between founders of resources and recruits in other insects, emphasizing the need for more detailed studies such as those conducted with birds and primates (e.g. Clay et al. 2012; Szimpl et al. 2015). Variation in the types and rates of signals by participants during recruitment may serve a number of functions, including communicating levels of motivation, calling to additional recruits, species or kin recognition, resource localization, or resource quality.

Alternative hypotheses for group formation mechanisms

Our results support the hypothesis that vibratory signals mediate group formation, but it is important to recognize that locating a shelter and feeding site is likely a complex process involving a series of behaviours and different sensory modalities. While searching for a shelter site, a larva first typically wanders along the leaf edge, and upon approaching, a

resident in a shelter may experience not only vibratory signals but also chemical (olfactory or gustatory), visual, or other forms of mechanical stimuli. We consider alternative or complementary mechanisms based on comparisons with other larval insects. Recruitment to food sources by means of chemical trails is the most commonly reported for caterpillars, but in all reported examples, these are central-place or nomadic foragers that move together to new food sources or move back and forth from the food source to the shelter (Costa and Pierce 1997; Costa 2006). Because early-instar *D. arcuata* are patch-restricted foragers (i.e. remain in the same patch), chemical trails would not be likely because they feed within the shelter. It is possible that olfactory cues or pheromones deposited on the silk or in frass attached to the shelter are detected by potential recruits. Vision seems to be an unlikely mechanism involved in recruitment, as larvae possess fairly simple optical systems (Warrant et al. 2003). However, visual cues may be involved in the searching process, such as detecting the leaf edges during wandering (Gilbert 1994). We rule out the involvement of tactile cues in *D. arcuata* larvae because there was no physical contact observed between larvae during recruitment events. We conclude that vibratory signals play an important role as recruitment signals in *D. arcuata* but surmise that other signals or cues, such as pheromones in the silk shelter or frass, may also contribute to group formation.

Alternative hypotheses for vibratory signalling

Our results support the hypothesis that vibratory signals advertise food and shelter resources to conspecifics. However, alternative hypotheses explaining the functions of these signals should be considered. One hypothesis is that vibrations function as distress signals to recruit help (Cocroft 1996; Travassos and Pierce 2000). However, there is no

evidence at present to support this hypothesis in *D. arcuata*. First, when disturbed by plucking at the shelter to simulate an invertebrate predator, larvae become silent rather than increase their signal rates, and there is no evidence of group antipredator defenses such as dropping, regurgitation, thrashing, or flicking (Matheson 2011; unpublished data JEY). Second, *D. arcuata* larvae are not tended by ants, and parental care is absent. Another hypothesis is that vibratory signals could be used to enhance feeding by a vibratome effect as observed in ants (Tautz et al. 1995), but this is not possible in *D. arcuata* as chewing does not coincide directly with AS or any of the other signals. Another hypothesis is that signals function as territorial signals. If so, it would be predicted that (1) R would generate signals only when approached by an intruding conspecific, and this is not the case, as R signals even in the absence of a conspecific, and (2) the approaching conspecific would not join and share the shelter with the resident larva, and we have shown that joining occurs following signalling of the resident. While our results do not support the hypothesis that signalling functions as a territorial defense signal by excluding a conspecific from the shelter, it remains possible that within the group, larvae maintain their own territories. However, this remains to be tested experimentally and would require analysis of established groups of two or more individuals, which was beyond the scope of the current study.

4.7 Conclusions

Caterpillar social groups are diverse and exhibit complex interactions between conspecifics for purposes of foraging, defense, and shelter construction (Costa and Pierce 1997). Yet, like for many of the “other social insects,” there is still a dearth of information on

communication (Costa 2006). Scientists are only beginning to appreciate the importance of vibratory communication in insects, which is believed to be particularly important for close-range interactions in juvenile insects, such as caterpillars, which are substrate-bound (Cocroft and Rodriguez 2005; Yack 2016). Cocroft and Hamel (2010) proposed that vibratory recruitment to feeding sites is probably widespread in the “other insect societies,” but at present, there are few documented examples. Our study provides the first example in caterpillars. However, we anticipate that vibratory communication in early-instar *D. arcuata* functions beyond group recruitment. In the current study, we focused on the role of vibratory signals only during the initial stages of group formation. However, once groups are formed, complex vibratory interactions occur between individuals and these interactions are hypothesized to play roles in division of labor, shelter construction, taking turns to feed, spacing, and orientation (Matheson 2011). The importance of vibratory signals and cues remains poorly documented for most group-living insects, and future studies should combine detailed behavioural observations with experimental manipulations and playback studies to gain a full appreciation of the rich vibratory landscapes of these insects.

Chapter 5: Transcriptome analysis of a social caterpillar, *Drepana arcuata*: de novo assembly, functional annotation and developmental analysis

This chapter is an adaptation of the following publication:

Yadav, C., Smith, M.L., & Yack, J.E. (2020). Transcriptome analysis of a social caterpillar, *Drepana arcuata*: De novo assembly, functional annotation and developmental analysis. *PLoS ONE*, 15(6), Article e0234903.

Statement of contribution:

C. Yadav collected the data, performed the analyses, prepared the figures and wrote the paper; M.L. Smith helped with molecular, genomics work, and editing; J.E. Yack supervised the project, contributed to figures, writing and editing.

5.1 Abstract

The masked birch caterpillar, *Drepana arcuata*, provides an excellent opportunity to study mechanisms mediating developmental changes in social behaviour. Larvae transition from being social to solitary during the 3rd instar, concomitant with shifts in their use of acoustic communication. In this study we characterize the transcriptome of *D. arcuata* to initiate sociogenomic research of this lepidopteran insect. We assembled and annotated the combined larval transcriptome of “social” early and “solitary” late instars using next generation Illumina sequencing, and used this transcriptome to conduct differential gene expression analysis of the two behavioural phenotypes. A total of 211,012,294 reads generated by RNA sequencing were assembled into 231,348 transcripts and 116,079 unigenes for the functional annotation of the transcriptome. Expression analysis revealed 3300 transcripts that were differentially expressed between early and late instars, with a large proportion associated with development and metabolic processes. We independently validated differential expression patterns of selected transcripts using RT-qPCR. The expression profiles of social and solitary larvae revealed differentially expressed transcripts coding for gene products that have been previously reported to influence social behaviour in other insects (e.g. cGMP- and cAMP- dependent kinases, and bioamine receptors). This study provides the first transcriptomic resources for a lepidopteran species belonging to the superfamily Drepanoidea, and gives insight into genetic factors mediating grouping behaviour in insects.

5.2 Introduction

Sociality is key to the success of many insects (Wilson 1985; Costa 2006; Costa 2018). The ultimate benefits associated with sociality, including enhanced foraging, predator defense, disease resistance and increased survival, have been well studied (e.g. Wilson 1985; Costa 2006; Costa 2018; Cremer et al. 2018). Proximate mechanisms mediating social behaviours such as group formation, division of labour, and foraging have been studied at different levels of analysis, including hormonal, neural, sensory and genetic (e.g. Miura 2004; Zayed and Kent 2015; Leonhardt et al. 2016; Kamhi et al. 2017). Over the past two decades, the advent of genomic resources and tools has led to immense progress in the field of sociogenomics, a discipline that focuses on the molecular genetic basis of sociality (Robinson et al. 2005). Within this field, comparative approaches have led to the identification of genes or gene products associated with social behaviours. One approach is to compare different species or higher-level taxa exhibiting similar or different social behaviours. For example, a comparative sociogenomic analysis using honeybee (*Apis mellifera*), fire ant (*Solenopsis invicta*), and paper wasp (*Polistes metricus*) transcriptomes was conducted to understand the genetic underpinnings of caste development (Berens et al. 2014). The results suggested that shared molecular pathways and biological functions mediate caste development in these three eusocial insect lineages. Such studies rely on analyses of genes or pathways that are conserved across the taxa and may not reveal species or lineage-specific novel genes involved in sociality. An alternative approach is to compare the genetic bases of different social behaviours within a species. Analysis of temporal polyethism (i.e. developmental changes in behaviour) in honeybees (*A. mellifera*), where workers transition from nurses to foragers, is a good example of such an intraspecific

approach. Using microarray analyses of RNAs expressed in worker bee brains, researchers identified differentially expressed genes associated with age polyethism (Kucharski and Maleszka 2002; Whitfield et al. 2003). Similarly, in locusts (*Locusta migratoria*, *Schistocerca gregaria*), more than 200 differentially expressed genes were correlated with the transition between solitary and gregarious phases (Chen et al. 2010; Badisco et al. 2011). By comparing within species, we can better understand how differential regulation of a particular gene or set of genes can mediate changes in social behaviours.

A developmental shift in grouping behaviour is observed in a number of arthropods, including spiny lobsters, spiders, sawfly larvae, and larvae of several lepidopteran species (Costa and Pierce 1997; Ratchford and Eggleston 1998; Anstey et al. 2002; Costa 2006; Moughenot et al. 2012). Benefits incurred from developmental changes in sociality may be related to shifts in competition for resources, foraging efficiency, thermoregulation, and predator defense (Costa 2006; Despland and Huu 2007; McClure and Despland 2011). Proximate mechanisms underlying such ontogenetic shifts are poorly understood, with limited research conducted to date. To the best of our knowledge, no genomic approaches have been undertaken to study developmental shifts in grouping behaviour in arthropods. The masked birch caterpillar (*Drepana arcuata*, Drepanoidea) presents an excellent opportunity to explore such mechanisms. The caterpillar transitions from a social to solitary lifestyle during development (Yadav and Yack 2018 [see Chapter 2]) (Figure 5.1), and the complex acoustic communication systems that mediate these social interactions have been studied in this species and its relatives (e.g. Yack et al. 2001; Bowen et al. 2008; Guedes et al. 2012; Yack et al. 2014; Yadav et al. 2017 [see Chapter

2]). The first two instars (I, II) are referred to as “early instars”, the fourth and fifth (IV,V) as “late instars” and the third (III) as the “transitional instar”. Eggs are laid in rows and neonates form small social groups that exhibit vibratory-mediated social interactions in shared silk shelters (Yadav et al. 2017 [see Chapter 4]; Yadav and Yack 2018 [see Chapter 2]) (Figure 5.1). Larvae remain in groups until third instar, when they transition to a solitary lifestyle. Late instars exhibit vibratory-mediated territorial behaviour to defend solitary silk leaf shelters (Yack et al. 2001; Yack et al. 2014). We propose the masked birch caterpillar is highly suitable for sociogenomic research because: (i) it transitions in social grouping behaviour at a predictable time— the third instar; (ii) we have insights into the sensory-motor communication mechanisms mediating grouping and solitary behaviour; (iii) there is opportunity to conduct interspecies comparative genomic analyses, as different species of Drepanoidea exhibit varying levels of sociality (Scott 2012).

Figure 5.1 Selected developmental stages of *D. arcuata*. (A) Adult moth. Scale bar: 5 mm. (B) Eggs laid in rows. Scale bar: 1 mm. (C) Group of early (I) instars in a silk shelter on a birch (*Betula papyrifera*) leaf. Scale bar: 2 mm. (D) Solitary late (IV) instar in a silk shelter on a birch leaf. Scale bar: 5 mm.

This study takes an important step towards developing the masked birch caterpillar as a non-model organism for sociogenomic research, and provides the first genomic resource for any Drepanoidea species. There are three main goals: First, to assemble and annotate the larval transcriptome of *D. arcuata*. Second, to use this de novo assembled transcriptome as a reference to conduct differential gene expression analysis between “social” early and “solitary” late instars. Third, to identify the expression profiles of genes that are potentially involved in social interactions (i.e. “social” genes).

Data Availability Statement: Raw RNA-seq data used in this study is at NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA556910 (see Appendix Table B5.1 for SRA and BioSample accession numbers). The assembled transcriptome sequences are at DDBJ/EMBL/ GenBank under accession number GIKL000000000, the version described in this paper is the first version, GIKL010000000. The transcript expression data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (GEO) and are accessible through GEO Series.

5.3 Material and methods

5.3.1 Sample preparation

An overview of methods used in this study is shown in Figure 5.2. Larvae were reared from eggs laid by females captured at ultraviolet lights near Ottawa, ON, Canada (45.4215 °N, 75.6972°W). Caterpillars were reared on paper birch (*Betula papyrifera*) leaves held in water-filled vials contained in glass jars at room temperature (21–23°C) on a 16 h: 8 h light: dark cycle. Instars were identified based on their head capsule morphology (Yadav and

Yack 2018 [see Chapter 2]). Early and late instars used for RNA extraction were in groups and solitary in their shelters, respectively, when collected. No specific permits were required for the collection of moths for this study. This study did not involve any protected or endangered species.

For high-throughput transcriptome sequencing, larvae from the same female (female #1, Figure 5.2) were used for all RNA extractions. Three biological replicates for each of early instars (I, II; N = 20 larvae [a mix of approximately equal numbers of I & II] per replicate) and late instars (IV,V; N = 5 larvae [3:2 mix of IV & V] per replicate) were frozen separately in liquid nitrogen prior to RNA isolation (see below) for sequencing. Early instars are much smaller in body size (1.7–5.5 mm) than late instars (6.6–20 mm) (Yadav and Yack 2018 [see Chapter 2]) and therefore to obtain sufficient RNA, more early instars were required per replicate.

Figure 5.2 Summary of methods used for transcriptome assembly and differential transcript expression analysis. E1, E2, E3 and L1, L2, L3 refer to the 3 replicates of early and late instars, respectively.

5.3.2 *RNA extraction and sequencing*

Total RNA was extracted using a Norgen Biotek RNA extraction kit (Norgen Biotek Corp, Thorold, ON, Canada) from whole caterpillar bodies that were flash frozen in liquid nitrogen. Extracted RNA samples were assessed for quantity and quality using Qubit HS RNA kit (Thermo Fisher Scientific, Waltham, MA, USA) and Fragment Analyzer High Sensitivity RNA kit (Agilent, Santa Clara, CA, United States), respectively, and subjected to paired-end Illumina sequencing at the StemCore sequencing facility (Ottawa Hospital Research Institute, ON, Canada). At the facility, RNA sequencing libraries were generated using TruSeq RNA Library Prep Kit v2 (Illumina, San Diego, CA, USA) using manufacturer's protocol. The quality and quantity of each sample library were assessed using Agilent Fragment Analyzer (Agilent, Santa Clara, CA, USA) with the High Sensitivity NGS Fragment Analysis assay and Qubit Double Stranded DNA HS kit (Thermo Fisher Scientific, Waltham, MA, USA), respectively. RNA sequencing was performed on the Illumina NextSeq500 platform to generate 150 bp paired-end reads. Analysis described in the sections below were performed remotely on the Extreme Science and Engineering Discovery Environment (XSEDE) (Towns et al. 2014).

5.3.3 *De novo transcriptome assembly and annotation*

Raw sequences were quality checked using FastQC v0.11.7 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and trimmed for adapter sequences, poor quality, and short sequences (<50 bp) using Trimmomatic-0.36 (Bolger et al. 2014). The resulting clean reads from both early and late instars were then assembled into transcripts using de novo assembler Trinity v2.4.0 (Grabherr et al. 2011) with default parameters, including the

normalization step. Using Trinotate v3.1.0 (Bryant et al. 2017), transcripts were annotated with the following databases: nonredundant database (NR), SwissProt, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Clusters of Orthologous Groups for eukaryotes (KOG) using BLASTx at e-value $\leq 1e-5$ (Kanehisa and Goto 2000; Tatusov et al. 2000). Transdecoder 3.0.1 (<https://github.com/TransDecoder/TransDecoder/wiki>) was used to predict the open reading frames (ORFs), retaining the ORFs of ≥ 100 amino acids in length. Following annotation, the completeness of the transcriptome was evaluated using BUSCO v3.0 (Benchmarking Universal Single-Copy Orthologs) (<https://busco.ezlab.org>) (Simao et al. 2015).

5.3.4 *Differential transcript expression analysis*

Transcript expression level differences between early (I, II) and late (IV, V) instars was analyzed using differential gene expression tools included within the de novo assembler Trinityv2.4.0, with default parameters. The expression analysis included two steps: (1) transcript abundance estimation using RSEM (Li and Dewey 2011) and; (2) identification and counting of transcripts expressed differentially between early and late instars using edgeR (Robinson et al. 2010), at False Discovery Rate (FDR) ≤ 0.001 and log2 fold change ≥ 2 , followed by hierarchical clustering based on expression values.

5.3.5 *RT-qPCR validation of differentially expressed transcripts*

To validate the transcript expression data generated by RNA-sequencing and analysis, RTqPCR was performed with 10 arbitrarily selected genes (3 down-regulated and 7 up-regulated in early instar relative to late instar). RNA was extracted from frozen larvae, all

derived from a single female (female #2, Figure 5.2) different than that used in the RNA-seq analyses (N = 10–15 larvae for early instars with approximately equal numbers of instars I, II, N = 1 larva for instar IV or V; three biological replicates for each). First strand cDNA synthesis was performed (cDNA synthesis quick protocol of New England Biolabs Inc., NEB#M0253) using 1 µg RNA, quantified using Nanodrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). 100 ng of cDNA was used for each RT-qPCR reaction. Housekeeping gene elongation factor 1A (ef1a) was used as a reference. Primers for the 10 tested genes were designed using Primer3Plus (Untergasser et al. 2007) and sequences for the primers are provided in Appendix Table B5.2 . RT-qPCRs were performed using SYBR Fast Universal qPCR kit using three technical replicates for each of the three biological replicates, in a total reaction volume of 20 µl (10 µl SYBR mix, 2 µl each of 10 µM forward and reverse primers, 4 µl MQ water and 2 µl of cDNA). qPCR was carried out using the CFX Connect system (Bio-Rad Laboratories Inc., Hercules, CA, USA) with the following thermal cycling conditions: Initial denaturation at 95°C for 3 min followed by 40 cycles of 95°C for 10s, 60°C for 15s and 72°C for 20s.

Information on the data used in this paper can be found under the “Data Availability Statement”, and includes sequencing and assembly data at NCBI Sequence Read Archive (SRA) and DDBJ/EMBL/GenBank, respectively, and the transcript expression data at NCBI’s Gene Expression Omnibus (GEO) (Edgar et al. 2002).

5.4 Results and discussion

5.4.1 De novo transcriptome assembly and annotation

RNA samples and sequencing: A total of 259,401,581 paired end 150 bp raw reads were obtained from the combined RNA samples (Table 5.1). Quality assessment indicated that the average GC content was 48.5%, with a Phred quality score above 20 for 100% of the bases. Quality filtering and removal of reads below 50 bp resulted in a total of 211,012,294 high quality reads (81.5% of the raw reads, Phred score ≥ 33). Quality filtered reads were pooled from all replicates of early and late instars to generate a combined reference transcriptome assembly (see below).

De novo transcriptome assembly: A total of 231,348 transcripts (N50 = 2050; 116,079 unigenes) were generated (Table 5.1), where 31.58% of the transcripts were above 1 kb (Appendix Figure B5.1). Unigene here refers to the longest isoform per cluster of transcripts assembled by Trinity that share sequence content. This large number of transcripts relative to unigenes is normal for the Trinity assembler, as there could be a number of biologically relevant isoforms and paralogs (Grabherr et al. 2011). Therefore, instead of using only the longest isoform per gene (unigene) we used all the transcripts for downstream analysis. BUSCO assessment, which provides quantitative assessment of transcriptome completeness in terms of gene content (Simao et al. 2015), revealed that the assembly was 97.3% complete, with 2.5% fragmentation and 70.1% duplication. Again, high duplication values are expected, as we used all transcripts generated by Trinity for analysis. BUSCO assessment of unigenes indicated only a ~2% duplication rate. Individual transcriptome assemblies were also generated for early and late instars (Appendix Table B5.3). These were comparable to other larval Lepidoptera transcriptomes in terms of number of transcripts and N50 values (e.g. Cui et al. 2017; Peterson et al. 2019). For a better

representation of the complete larval transcriptome we combined the early and late instar sequences to assemble and annotate a reference transcriptome. This reference transcriptome was used to identify stage-specific RNA expression differences between early (social) and late (solitary) instars.

Table 5.1 Summary of RNA sequencing data and combined early and late instar larval transcriptome.

Sequencing data		
Total raw reads	259,401,581	
Total clean reads	211,012,294	
% GC	48	
Transcriptome assembly statistics		
	Transcripts	Unigenes
Total number	231,348	116,079
N50 length	2050	1190
Median length	487	333
Average length	1041.92	671.60
Total assembled bases	241,045,801	77,959,215
% GC	42.38	

Transcriptome annotation: Multiple databases were used to annotate the de novo assembled reference transcriptome (see annotation report in Appendix File B5.1). Using an e-value cut-off of $1e-5$, 112,661 transcripts (48.69%) were found to have significant homology to sequences in NCBI's non-redundant (NR) database. Of these, 74% of transcripts matched best to genes from lepidopteran species and the remaining 26% to other invertebrate species (Figure 5.3). GO terms, KEGG and KOG annotations were assigned. A total of 75,023 transcripts (32.42%) were assigned to 21,062 GO terms, whereas ~67% of transcripts were of unknown function, highlighting a knowledge gap in our understanding of non-model insect genomes. The major GO categories were biological process (69.87%), cellular component (9.2%), and molecular function (20.85%). Similar to other larval Lepidoptera, biological regulation and metabolic process (in biological process), catalytic activity and binding (in molecular function) and cell and cell part (in cellular component) were amongst the top subcategories represented (Figure 5.4) (e.g. Cui et al. 2017a, b). A total of 19,803 transcripts (8.5%) were annotated to KEGG pathways, divided into 5 categories (Appendix Figure B5.2), and 49,918 putative proteins (predicted by TransDecoder) were assigned to 4395 KOG terms, classified into 26 KOG groups (Appendix Figure B5.3). Similar to other larval lepidopterans (Cui et al. 2017a, b; Peterson et al. 2019) metabolic pathways were most represented in KEGG annotations, and general function only in KOG annotations.

Figure 5.3 Homology of transcripts to Non-Redundant (NR) protein database. Percentages indicate the distribution of best matches for *D. arcuata* transcripts to those of other invertebrates based on the homology search conducted with NR db at an e-value cut off of $1e-5$. Top 5 hits were Lepidoptera-*Helicoverpa armigera*, *Spodoptera litura*, *Heliothis virescens*, *Bombyx mori* and *Amyelois transitella*.

Figure 5.4 Gene Ontology (GO) classification of larval transcriptome. Transcripts were classified into three functional categories based on GO annotation- (A) biological process, (B) cellular component, and (C) molecular function.

Lepidoptera are one of the largest insect orders, with >157,000 described species (Triant et al. 2018). Many species are of economic significance as forest and agricultural pests, and ecologically significant as pollinators and food sources (Goldstein 2017). Additionally, many species are used as model organisms for studies in ecology, evolution and physiological processes (e.g. Roe et al. 2010; Wallbank et al. 2016; Pearce et al. 2017; Borzoui et al. 2018). While genomic information is increasingly being applied to this research, there is a clear lack of genomic resources for most lepidopteran superfamilies (Triant et al. 2018). Our study provides the first transcriptomic resources for a species belonging to the superfamily Drepanoidea. The Drepanoidea comprises >1400 species distributed throughout the world (Minet and Scoble 1999). This group includes species of interest for their unique hearing organs (Surlykke et al. 2003), as pests of coffee (Hill 1983), and larval vibroacoustic communication (Yack et al. 2001; Bowen et al. 2008; Scott et al. 2010; Guedes et al. 2012; Scott 2012; Scott and Yack 2012; Yack et al. 2014; Yadav et al. 2017). Notably, due to variability in their social structure both between and within species, and their uniquely complex vibratory communication systems, larval Drepanoidea hold much promise for future research testing hypotheses on the function and evolution of communication and sociality. Our study takes a first step in facilitating this research by providing insights into the larval developmental transcriptome, including the identification of genes potentially associated with a behavioural shift in sociality.

5.4.2 *Differentially expressed transcripts in early vs late instars*

Based on RNA-seq, 3300 transcripts (3098 unigenes) were identified to be differentially

expressed at $\log_2FC \geq 2$ ($FDR \leq 0.001$) between early and late instars (Figure 5.5; Appendix File B5.2). Log₂FC here refers to the log₂ ratio of transcripts' expression values in late vs early instars. Transcript expression levels based on RNA-seq data were congruent with RT-qPCR results with 10 arbitrarily selected DETs ($R = 0.9710$; Appendix Figure B5.4), supporting our use of RNA-seq data to compare gene expression patterns between early and late instars. Of the 3300 DETs, ~34% were downregulated and ~66% were up-regulated in late instars relative to early instars. Examples of these DETs along with some of the most up-regulated transcripts in early and late instars are listed in Table 5.2. KEGG pathway analysis revealed that most of the differentially expressed transcripts are involved in metabolic pathways (40.14%), with carbohydrate metabolism, lipid metabolism, and amino acid metabolism representing the most represented subcategories. Also, these pathways were mostly upregulated in late instars relative to early instars (Appendix Figure B5.5). Metabolic processes were one of the top 3 represented categories in GO annotation as well (Appendix Figure B5.6). Differential expression of transcripts involved in metabolic processes could relate to developmental differences between early and late instars in mobility, feeding, acoustic signalling, silk production and nest building behaviours (Yadav and Yack 2018 [see Chapter 2]).

Figure 5.5 Heat map showing transcripts that are differentially expressed between early and late instars. E1, E2, E3 and L1, L2, L3 refer to the 3 replicates of early and late instars, respectively. Transcripts with differential expression values of $\log_2FC \geq 2$ at $FDR \leq 0.001$ were clustered together based on their expression patterns across the samples. The color (see color key on top left corner) indicates low (green) to high (yellow) expression values for transcripts. Dendrograms at top represent the clustering of early and late instar replicates based on their expression.

Table 5.2 Some of the most up-regulated transcripts and other interesting DETs in early and late instars.

Instar	<i>D. arcuata</i> Transcript ID	NR ID	NR ID annotation description	log ₂ FC*
Early	TRINITY_DN21660_c3_g1_i1	XP_022817426.1	juvenile hormone esterase-like	15.58
	TRINITY_DN22418_c3_g1_i31	XP_021188341.1	ribosome biogenesis protein WDR12 homolog	13.38
	TRINITY_DN17012_c1_g1_i1	XP_012552749.1	proteoglycan Cow	13.10
	TRINITY_DN22359_c0_g1_i6	XP_021189772.1	cytosolic carboxypeptidase 4-like isoform X1	12.18
	TRINITY_DN20585_c1_g1_i5	XP_022815245.1	histone-lysine N-methyltransferase trithorax isoform X2	12.16
	TRINITY_DN17657_c3_g4_i1	XP_004923731.1	ommochrome-binding protein	10.02
Late	TRINITY_DN20910_c0_g1_i5	XP_013165599.1	PREDICTED**: aminopeptidase N-like isoform X1	-4.25
	TRINITY_DN19503_c2_g2_i2	OWR48843.1	Serine protease H51	-6.42
	TRINITY_DN15158_c4_g1_i10	XP_013188410.1	PREDICTED**: cytochrome c oxidase subunit 6b-2-like	-7.40
	TRINITY_DN20861_c0_g1_i5	XP_021194029.1	mucin-5AC-like isoform X2	-9.74
	TRINITY_DN20396_c0_g1_i4	KOB76727.1	Cytochrome P450 6B46	-10.26
	TRINITY_DN15186_c1_g1_i2	XP_021188290.1	gamma-glutamyltranspeptidase 1-like isoform X2	-13.55
	TRINITY_DN18525_c2_g1_i1	NP_001040518.1	S-phase kinase-associated protein	-13.94
	TRINITY_DN21922_c0_g1_i1	XP_022831709.1	fatty acid synthase-like isoform X2	-14.39
	TRINITY_DN14155_c2_g1_i3	XP_013187092.1	PREDICTED**: glucose dehydrogenase [FAD, quinone]-like	-14.40
	TRINITY_DN21096_c3_g1_i4	NP_001139414.1	urbain precursor	-17.66

* +log₂FC values indicate upregulation in early instar, and -ve log₂FC values indicate upregulation in late Instar

**predicted function of respective (NR ID) homolog

Early instars ‘skeletonize’ birch leaves, feeding on the tender tissue between leaf veins, whereas late instars ingest entire sections of leaf. These feeding differences may account for our observation that digestive enzymes (e.g. mucin, serine protease) are among the relatively up-regulated DETs in late instars (Table 5.2). DETs encoding detoxification-related enzymes (cytochrome P450, aminopeptidase N, cytochrome C oxidase) were also up-regulated in late instars relative to early instars (Table 5.2). Detoxification enzymes are induced in insects in response to plant allelochemicals (e.g. Liu et al. 2006; Zeng et al. 2007). An increased expression of transcripts coding for the detoxification enzymes in late instars could be associated with increased feeding, hence enhanced detoxification of plant secondary metabolites and other toxins. As *D. arcuata* larvae develop they change in body color, from brownish-black in early to green in late instars (Yadav and Yack 2018 [see Chapter 2]). These striking coloration differences between early and late instars may relate to observed differential expression of pigmentation genes. For example, one of the DETs, ommochrome-binding protein, is suggested to influence larval body coloration in the silkworm, *Bombyx mori* (Kato et al. 2006; Okamoto et al. 2008) (Table 5.2).

Sociality-related genes: One of the goals of this study was to assess if there was differential expression of candidate genes for sociality between early and late instars. As defined by Fitzpatrick et al. (2005), candidate genes are those that have been identified to influence a certain phenotype in one organism, and are then tested for influencing a similar phenotype in another organism. Several orthologous genes appear to influence social behaviours across taxa, including, but not limited to, foraging, cooperative group living, mating and parental care. Some of the most studied “social” genes are listed in Table 5.3,

of which seven were identified among DETs between early and late instars of *D. arcuata* . Of particular interest are cGMP-dependent protein kinases, and biogenic amine-octopamine and dopamine receptors. Although a number of predicted “social” genes were identified among the *D. arcuata* DETs, there were some that were not observed to be significantly differentially expressed between early and late instars. These included syntaxin 1a, neuropeptide receptor (Npr)-, corazonin receptor, and vitellogenin receptor genes (Table 5.3).

Table 5.3 Examples of social genes/molecular products in invertebrates and their expression in larval *D. arcuata* transcriptome.

Gene/Product	Organism	<i>D. arcuata</i> transcript ID	log ₂ FC*
octopamine receptor	<i>L. migratoria</i> (Fitzpatrick et al. 2005)	TRINITY_DN15936_c1_g2_i1	4.90
dopamine receptor	<i>L. migratoria</i> (Ma et al. 2015)	TRINITY_DN21054_c1_g2_i1	4.86
takeout	<i>L. migratoria</i> (Guo et al. 2015)	TRINITY_DN19810_c0_g1_i3	4.82
period	<i>A. mellifera</i> (Guo et al. 2011)	TRINITY_DN20311_c0_g1_i6	4.51
NeuropeptideF precursor	<i>Drosophila melanogaster</i> (Bloch et al. 2001)	TRINITY_DN13765_c0_g1_i2	3.73
npr-1	<i>Caenorhabditis elegans</i> (Wu et al. 2003)	TRINITY_DN16480_c1_g1_i2	0.56**
corazonin receptor	<i>Harpegnathos saltator</i> (de Bono and Bargman 1998)	TRINITY_DN18580_c1_g2_i6	-0.198**
syntaxin 1a	<i>Lasioglossum albipes</i> (Gospocic et al. 2017)	TRINITY_DN21083_c1_g1_i14	-0.96**
vitellogenin, vitellogenin receptors	<i>A. mellifera</i> (Kocher et al. 2018)	TRINITY_DN14655_c2_g1_i1	-4.10**
cAMP-dependent protein kinase	<i>S. gregaria</i> (Nelson et al. 2007)	TRINITY_DN13079_c0_g1_i1	-7.15
for, cGMP-dependent protein kinase	<i>D. melanogaster</i> (Ott et al. 2012) <i>A. mellifera</i> (Osborne et al. 1997)	TRINITY_DN18355_c0_g1_i6	-9.61

* +log₂FC values indicate upregulation in early instar, and -ve log₂FC values indicate upregulation in late instar

** indicates log₂FC values at FDR>0.001, hence not included among DETs

cGMP-dependent protein kinase encoded by foraging (for) gene is implicated in the mediation of social and foraging behaviours in diverse insect orders including Hymenoptera, Orthoptera, and Diptera (Table 5.3). The natural allelic variation in the for gene influences fly phenotypes (rover vs. sitter), and the differential expression of the for gene is associated with nursing-foraging worker phenotypes in honeybees, ants and wasps, and gregarious-solitary phenotypes in desert locusts. For example, in honeybees there is an increased expression of cGMP-dependent kinases (encoded by for gene) in foragers that go out of the hive relative to the nurse bees that stay in the hive. Similarly, fly larvae that express the rover phenotype (carrying rover allele at for gene) forage over longer distances than sitters (carrying sitter allele at for gene). In *D. arcuata*, we observed relative upregulation of transcripts coding for the for ortholog in late instars ($\log_2FC = -9.969$, Table 5.3). As the *D. arcuata* larvae develop from early to late instars, they disperse from the communal silk shelters to forage individually and establish solitary leaf shelters. Therefore, the relative increase expression of cGMP-dependent kinase in late instars could be correlated with this change in foraging pattern, similar to what has been observed in rover phenotypes in flies and honeybee foragers (Osborne et al. 1997; Ben-Shahar et al. 2002).

Other interesting DETs to highlight are those coding for the biogenic amine receptors, dopamine (DA) and octopamine (OA). Biogenic amines have long been known to influence sociality and related behaviours in different insects such as locusts, ants, and honeybees (Wada-Katsumata et al. 2011; Guo et al. 2015; Ma et al. 2015; Hewlett et al. 2018). Particularly in locusts, some OA and DA receptors have been found to be associated with

gregarious-solitary state transitions (Guo et al. 2015; Ma et al. 2015; Xu et al. 2017). In *D. arcuata* larvae, we observed a relative upregulation of both OA and DA receptors in early instars that live in groups, relative to the late instars that live solitarily (Table 5.3). This differential expression of OA and DA could be associated with group formation and maintenance in early instars, similar to specific DA and OA receptors that mediate gregarious behaviour in locusts.

Our transcriptome analysis thus provides leads into genes that may influence the developmental shift from social to solitary in *D. arcuata* larvae, and the putative role of these genes can be further tested using pharmacological and/or genetic techniques such as RNAi and CRISPR. RNAi and CRISPR have been used with insects to study genetic modulation of different behaviours, including gregarious behaviour in the desert locust (Ott et al. 2012), nurse to forager transition in honeybees (Ament et al. 2012), migration in the monarch butterfly (Markert et al. 2016), and sociality in the ponerine ant (Trible et al. 2017; Yan et al. 2017).

5.5 Conclusion

This study provides the reference transcriptome for larval *D. arcuata*. As the first transcriptomic resource for a species within the superfamily Drepanoidea, this research fills a knowledge gap in the field of Lepidoptera genomics. Also, differential transcript analysis using RNA-seq data from social “early” and solitary “late” instars revealed a marked shift in the transcriptome profile, including changes in the expression of sociality-related genes involved in foraging and grouping behaviours in other insects. The masked

birch caterpillars and their relatives, that vary in social grouping behaviour within and between species, offer a great opportunity to explore proximate mechanisms of sociality in larval insects.

**Chapter 6: Studying the effects of a candidate “social” gene
(octopamine receptor) on the grouping behaviour of early instar *D.*
arcuata using RNAi: a preliminary investigation**

Statement of contribution

C. Yadav collected and analyzed the data, and wrote the chapter, including the figures and tables; M.L. Smith contributed to the project design, molecular data collection and editing; J.E. Yack supervised the project.

6.1 Abstract

Group living is observed across diverse insects, including larval Lepidoptera, and there is a growing interest in identifying the genetic underpinnings of associated social behaviours. *Drepana arcuata* is an excellent organism to explore genetic influences on social behaviour since the larvae predictably transition from social group living to solitary behaviour during the 3rd instar of development. Previously, a remarkable shift in the *D. arcuata* larval transcriptome was observed to correlate with the transition from social to solitary behaviour. The current study explores the genetic basis of sociality in *D. arcuata* by testing the function of one of the putative sociality-related genes – the octopamine receptor gene. I conducted preliminary investigations into octopamine receptor gene function by altering its expression using RNA interference (RNAi). Three dsRNAs matching different regions of the octopamine receptor gene were chemically synthesized and fed to second instar *D. arcuata* larvae. RT-qPCR results indicated a significant reduction in octopamine receptor transcript abundance in the dsRNA fed larvae compared to control. Behavioural trials indicated that reduced octopamine receptor transcript abundance is associated with expedited social to solitary behavioural transition in the larvae. Results of this preliminary study support the hypothesis that regulation of the octopamine receptor gene influences caterpillar sociality.

6.2 Introduction

Sociality is a commonly observed phenomenon in juvenile insects, and one aspect of sociality, group-living, is particularly widespread in larval Lepidoptera (caterpillars) (Costa 2006). There are >300 species of Lepidoptera spanning over 20 families that exhibit group-living during some stage of larval development (Costa and Pierce 1997). Potential benefits associated with group living in caterpillars include thermoregulation, predator defense, and feeding facilitation (Costa 1997; Costa and Pierce 1997; Prokopy and Roitberg 2001; Costa 2006; Fordyce 2006; Despland and Lee Huu 2007; Despland 2019). With respect to human affairs, group-living often contributes to the success of lepidopteran species as pests. Despite these considerations, mechanisms underlying group formation and cohesion are poorly understood in lepidopteran species. In fact, to my knowledge, only chemical communication has been thoroughly explored as an underlying mechanism of group living in caterpillars (Costa 2006).

Comparative genetic approaches hold great promise for unravelling the bases of social grouping mechanisms in caterpillars. Understanding how changes in expression of genes influence caterpillar social behaviour is now facilitated by the rapid growth in knowledge of Lepidoptera genomics. Using comparative approaches, specific orthologous genes are reported to mediate sociality in different organisms, implying conservation of gene function across species (Robinson et al. 2005; Reaume and Sokolowski 2011; Berens et al. 2014; Shell and Rehan 2019). Further comparative genetic approaches will lead to in-depth understanding of the mechanisms that underly group living behaviours and may lead to novel control strategies for larval Lepidoptera that are pest species. In this chapter, I will

conduct preliminary experiments on genetic mechanisms of sociality in Lepidoptera using the masked birch caterpillar, *Drepana arcuata* (Lepidoptera: Drepanoidea).

The masked birch caterpillar occurs throughout northern and east-southeastern North America (Rose and Lindquist 1997), feeding primarily on birch and alder leaves during larval stages. There are five instars reported for the species; early instars (I,II) that exhibit social group living behaviour, whereas late instars (IV,V) are solitary, and third instars (III) that transition from social to solitary (Yadav and Yack 2018 [see Chapters 2 and 3]). My recent transcriptomic work on *D. arcuata* caterpillars revealed changes in gene expression that may be related to a transition from social to solitary behaviours. Of particular interest are several differentially expressed genes that were previously shown to be associated with sociality in other organisms (Yadav et al. 2020 [see Chapter 5]). As a next step, I initiated experiments to test whether differential expression of one candidate gene, an octopamine receptor gene, is associated with the switch between behavioural states during development of *D. arcuata* larvae.

Octopamine is one of several biogenic amines known to play a key role in a variety of insect behaviours (Roeder 1999, 2005; Stern 1999; Cohen et al. 2002; Fussnecker et al. 2006; Dierick 2008) including social behaviour of eusocial insects (e.g Schulz et al. 2002; Wada-Katsumata et al. 2011; Cunningham et al. 2014; Hewlett 2018). Apart from eusocial insects, an interesting example of octopamine's influence is observed in locusts (*Locusta migratoria*), where octopamine receptor signalling is associated with the behavioural switch from repulsion to attraction (Ma et al. 2015).

While gene expression analysis can provide us with insights into the genetic changes associated with sociality, it does not allow unambiguous determination of whether a correlated change in gene expression is a cause or an effect of the behavioural switch. Insights into the underlying genetic pathways of behaviours can be gained by altering the expression of a target gene using gene knockdown or gene knockout techniques such as RNAi or CRISPR, respectively. By experimentally manipulating gene expression we can test the function of putative social gene(s) that were previously identified by transcriptomics analyses (Novina and Sharp 2004; Hunt and Gadau 2016).

The use of RNA interference (RNAi) technology can elicit a relatively temporary reduction in gene expression that may be lost within the same generation or over a few generations, and does not generally result in modification to the genetic code (Boettcher and McManus 2015; Moreira et al. 2020). CRISPR is a relatively new gene editing technique that can result in permanently altered gene expression or loss of gene function that is transferred over subsequent generations. Several studies have used CRISPR techniques with Lepidoptera (e.g., Koutroumpa et al. 2016; Ye et al 2017; Zhang and Reed 2017; Wang et al. 2020). In the current chapter, I attempted to modify gene expression using RNAi, and to test whether or not a change in expression affects the switch from social to solitary behaviour in *D. arcuata*. I chose an RNAi approach for two main reasons. First, RNAi methods can be used with caterpillars derived from wild caught female *D. arcuata*. In contrast, use of CRISPR usually entails several rounds of backcrossing to obtain the gene mutation in a homozygous condition. We have observed that *D. arcuata* suffers reduced

fitness with inbreeding in the laboratory (Yack lab, unpublished). Second, RNAi is likely to result in temporary down regulation of a target gene (knockdown), as opposed to CRISPR that is more likely to result in complete inactivation of the target gene (knockout). In this case, I was concerned that a CRISPR-associated loss of function of the octopamine receptor gene may have deleterious pleiotropic effects on the insect.

RNAi is a natural process in eukaryotes that can be utilized as a gene knockdown technique. Degradation of endogenous target RNA can be achieved by introducing into an organism exogenous (synthetic) double stranded RNA (dsRNA) that has sequence identity to the target gene. RNAi results in the degradation, and thus reduction, of transcript abundance of the target gene (Fire et al. 1998; Joga et al. 2016). Different types of small RNAs that trigger gene silencing in insects include short interfering RNA (siRNA), endogenous siRNA (endo-siRNA), microRNAs, and piwi-interacting RNA (piRNAs) (Brodersen and Voinnet 2009; Siomi and Siomi 2009a, b). RNAi has been widely used across Lepidoptera for almost two decades in more than 25 species across 10 families to study function of various genes associated with different biological processes (Xu et al. 2016). A majority of these studies have been performed in pest species (reviewed in Terenius et al. 2011; Xu et al. 2016). Methods employed to elicit RNAi by introducing dsRNA in Lepidoptera include microinjection, feeding, soaking, transgenesis, electroporation, virus- and bacteria-mediated uptake, and plant-mediated dsRNA uptake (reviewed in Xu et al. 2016). Among these methods, feeding dsRNA is one of the easiest and most natural ways to elicit RNAi without damaging the insect (reviewed in Xu et al. 2016). Some examples of successfully eliciting RNAi by feeding dsRNA among Lepidoptera include studies with diamondback

moth *Plutella xylostella*, beet armyworm *Spodoptera exigua*, tobacco hornworm *Manduca sexta*, european corn borer *Ostrinia nubilalis*, cotton bollworm *Helicoverpa armigera*, and gypsy moth *Lymantria dispar* (Terenius et al. 2011; Xu et al. 2016; Choi and Vander Meer 2019).

The main goals of this chapter were twofold. First, I set out to determine whether or not feeding synthetic dsRNA to *D. arcuata* larvae would elicit an RNAi response. This would be evident by a reduction in octopamine receptor gene transcript abundance based on RT-qPCR analysis. Second, if downregulated, I set out to determine whether a reduction in octopamine receptor gene transcript abundance alters the timing of social to solitary transition in *D. arcuata* caterpillars. Previously, transcriptomic analyses of social and solitary *D. arcuata* larvae showed an upregulation of the octopamine receptor gene in socially-grouping early instars relative to the solitary living late instars (Yadav et al. 2020 [see Chapter 5]). Based on this observation, I hypothesize that RNAi-induced downregulation of the octopamine receptor gene in 2nd instar caterpillars would hasten the switch from social to solitary behaviours.

6.3 Materials and Methods

6.3.1 Insect collection and rearing

Larvae were reared from eggs laid by female *D. arcuata* moths collected at locations close to Ottawa, Ontario, Canada (45.4215 °N, 75.6972 °W). Eggs were laid on paper birch, *Betula papyrifera*, and neonates were carefully transferred to fresh birch leaves using a fine

paintbrush. Larvae were reared on birch leaves held in water-filled plastic vials kept in glass jars, and kept indoors at room temperature (21–23°C and 16 h: 8 h light:dark).

6.3.2 *dsRNA design and synthesis*

Based on larval transcriptomic analysis, isoforms of the octopamine receptor gene were found to be upregulated in socially grouping early instars of *D. arcuata* relative to the solitary late instars (Yadav et al. 2020 [see Chapter 5]). ‘Isoforms’, here, refers to multiple full-length transcripts that can result from alternative splicing or paralogous genes, and were assigned to octopamine receptor gene by the transcriptome assembler, Trinity (Grabherr et al. 2011; Yadav et al. 2020 [see Chapter 5]). Double-stranded siRNAs were designed to target three different regions of octopamine receptor transcript (see sequences in Table 6.1; Figure 6.1). Isoforms of octopamine gene were first aligned using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) and regions of identity among the isoforms (Figures 6.1, Appendix C6.1) were used for dsRNA design using Dharmacon’s siDESIGN tool (<https://horizondiscovery.com/en/products/tools/siDESIGN-Center>). Each siRNA (21-nucleotide long) was then commercially synthesized by Sigma Aldrich (<https://www.sigmaaldrich.com/life-science/custom-oligos/sirna-oligos.html>), with a dTdT overhang at the 3’ end that is recommended to enhance nuclease resistance of siRNAs (Elbashir et al. 2001). For preparing dsRNA, each sense and antisense siRNA (dry-supplied by the manufacturer) were separately dissolved in 100 µl of diethyl pyrocarbonate-treated water (DEPC water) as per manufacturer’s instructions and then complementary pairs of siRNAs were combined together to allow formation of duplex RNAs (referred to as dsOAR in the following sections) at a final concentration of 1.5 µg/µl.

Table 6.1 Commercially synthesized siRNAs for *D. arcuata* octopamine transcript sequence.

siRNA	Location relative to ATG start codon	Nucleotide sequence
dsOAR-1		
Sense	83-103	5'- GUACCUCGUAUGCCCUCUAUA[dT][dT] -3'
Anti-sense	103-83	5'- UAUAGAGGGCAUACGAGGUAC[dT][dT] -3'
dsOAR-2		
Sense	740-760	5'- GGAUCCUAUCGCUUGUCAUUA[dT][dT] -3'
Anti-sense	760-740	5'- UAAUGACAAGCGAUAGGAUCC[dT][dT] -3'
dsOAR-3		
Sense	1467-1487	5'- GCUCCCAUUCUUCGUGAUUUA[dT][dT] -3'
Anti-sense	1487-1467	5'- UAAAUCACGAAGAAUGGGAGC[dT][dT] -3'

Figure 6.1 Diagram representing the octopamine receptor (OAR) transcript, the locations of three dsRNAs used for RNAi experiments (top) and primers used for RT-qPCR. Thick arrow represents the coding sequence of octopamine receptor transcript.

6.3.3 *dsRNA feeding and behavioural trials*

Birch leaves of approximately 4-6 cm² were suspended by their petioles in water-filled Eppendorf tubes for each trial. Using a p1000 micropipette tip, 3 µg dsRNA (i.e. 2 µl volume) was applied evenly per 1 cm² area of the top surface of the leaf (treated), and an equal volume of DEPC water was applied to the top surface of control leaves. Each dsRNA was used separately for the trials, and 2nd instar caterpillars, identified by head capsule size and other morphological features (Yadav and Yack 2018 [see Chapter 2]) and collected within 1 day of molting, were used for the trials in order to avoid any molting to 3rd instars (the developmental time for 2nd instars is ~4 days; Yadav and Yack 2018 [see Chapter 2]). One hour after application of dsRNA (treated) or DEPC water (control), 6-9 2nd instars were placed on the leaves and allowed to feed on these leaves for 48 hours. After 48 hours, larvae were removed from treated and control leaves, and further used for behavioural trials to assess if the dsRNA treatment affected their group forming behaviour. Group formation trials were performed by placing the larvae spatially separated on 2-3 leaves (each leaf = ~8 cm long x ~5 cm wide) attached to a birch twig held in water-filled plastic vial, and group formation by the larvae over a period of 24 hours was recorded (see Figure 6.2). Grouping data were subjected to one-way analysis of variance (ANOVA) and Tukey's HSD using R-package 'haven v2.3.1' in RStudio v1.3.1056 (R Core Team 2019).

Figure 6.2 An overview of methods employed in the current study testing the influence of an octopamine receptor gene using RNAi on the social behavioural transition in *D. arcuata* caterpillars.

6.3.4 Primer design and RT-qPCR

6.3.4.1 Primer design

Primers for a ‘housekeeping gene’, *rps7* (ribosomal protein 7), and octopamine receptor gene were designed using Primer3Plus v2.4.2 software (Untergrasser et al. 2007). Sequences of these genes were retrieved from *D. arcuata* transcriptome assembly deposited at DDBJ/EMBL/GenBank under accession number GIKL000000000 (Yadav et al. 2020 [see Chapter 5]) to design primers (see Table 6.2 for primer sequences).

Table 6.2 Sequences of octopamine receptor gene, OAR, primers and housekeeping gene, *rps7*, primers used for RT-qPCR.

OAR

F	5'- TTCCGAGGTGTACGAGGTAA-3'
R	5'- ACATATCGCTTCCCATTCTG-3'

rps7

F	5'- GTTGAAGGCATTCCAGAAGA-3'
R	5'- TAGGGAGAATCTTGCGGTCT-3'

6.3.4.2 *RNA extraction and cDNA synthesis*

A subset of two to three 2nd instar individuals from each RNAi trial were used for RNA extractions (Figure 6.2). For this, caterpillars were flash frozen in liquid nitrogen at each of three time points during trials: 0 h (start of trials), 48 h (end of dsRNA feeding), and 72 h (end of behavioural observations) (see Figure 6.2 for workflow). Frozen caterpillars were then stored at -80 °C prior to RNA purification. Frozen caterpillars were ground in liquid nitrogen using a mortar and pestle followed by lysis and RNA purification using Norgen Biotek Plant/Fungi RNA extraction kit (#31350). DNase (Invitrogen TURBO DNase, 2 U/μl) was added during RNA extraction to eliminate residual DNA. Isolated RNA was quantified using Nanodrop Spectrophotometer and stored at -80 °C in aliquots until cDNA synthesis. One μg of RNA was used for cDNA synthesis using M-MuLV reverse transcriptase following First Strand cDNA Synthesis (Quick Protocol) from New England Biolabs (NEB #M0253).

6.3.4.3 *RT-qPCR*

RT-qPCRs were performed using SYBR Fast Universal qPCR kit. qPCR reactions were performed in duplicate with a total reaction volume of 20 μl (10 μl SYBR mix, 2 μl each of 10 μM forward and reverse primers, 4 μl MQ water and 2 μl of cDNA). qPCR was carried out using Bio-Rad's CFX Connect system (Bio-Rad laboratories, Hercules, California, USA) with the following thermal cycling conditions- Initial denaturation at 95 °C for 3 min followed by 40 cycles of 95 °C for 10s, 60 °C for 15s and 72 °C for 20s. No template control (NTC) and no reverse transcriptase control (NRT) were used during each

qPCR reaction to confirm the absence of primer-dimer formation and DNA contamination, respectively. RT-qPCR values across trials were normalized to the reference gene (*rps7*) and the double delta Ct method (Livak and Schmittgen 2001) was used for relative quantification of octopamine transcript abundance in treated vs control caterpillars.

6.4 Results and discussion

The two main objectives in the current chapter were to determine: 1) whether feeding synthetic dsRNA to *D. arcuata* larvae would reduce transcript abundance of the octopamine receptor gene, and (2) whether a reduction in octopamine receptor transcript abundance alters the timing of the social to solitary transition.

6.4.1 Alterations in octopamine receptor transcript expression

Three dsRNAs (dsOAR-1, dsOAR-2, dsOAR-3) that target different regions of octopamine receptor transcript were each fed to *D. arcuata* larvae to examine for evidence of RNAi (Table 6.3). The estimated area of leaf consumed by each larva (assuming equal feeding by all larvae during trials), both in control and treatment trials, was similar in ranging from 1.5-3 mm². This is equivalent to a dose of 0.45-0.9 µg of dsRNA per larva, again assuming equal feeding by all larvae during trials. RT-qPCR results revealed a significant reduction of target octopamine receptor transcript abundance at 48 h using both dsOAR-2 and dsOAR-3, and a non-significant reduction in the transcript levels when dsOAR-1 (see Table 6.3). Subsequent trials were conducted using only dsOAR-2 and dsOAR-3. Also evident in Table 6.3 is that octopamine receptor transcript abundance appears to increase by 72h with all three dsRNAs used. This suggests that dsRNA knockdown of octopamine

receptor transcript occurs following administration of dsOAR-2 and dsOAR-3, as evident at 48 h, but the effect is transitory and much reduced by 72 h (i.e. 24 h after end of dsRNA treatments). Overall, the combined results from all trials with dsOAR-2 and dsOAR-3 indicate that 48 h of feeding dsRNA-coated birch leaves results in a significant reduction the target octopamine receptor transcript abundance, compared to caterpillars fed DEPC water-coated control leaves ($p < 0.001$; Figure 6.3).

Figure 6.3 Octopamine receptor transcript abundance after 48 h of dsOAR-2 and dsOAR-3 treatments or DEPC water controls. 2nd instars were allowed to feed on dsOAR-coated leaves (treated) or DEPC water (control) leaves for 48h and octopamine gene expression was quantified using RT-qPCR. Compared with the control, a significant decrease in the transcript abundance levels of octopamine receptor gene was observed (unpaired t-test, $P < 0.0001$).

6.4.2 Group formation trials

To determine if the reduced octopamine receptor transcript abundance inferred from RT-PCR results in changes in the behavioural transition from social to solitary in *D. arcuata* larvae, group formation observations were made during the 48 – 72 h period of trials. Results show that 78.26% of the larvae fed with dsOAR-2 and dsOAR-3 dsRNA remained solitary throughout the 24 h observation period (Table 6.3; Figure 6.4), with a significant difference in the number of larvae that became solitary between dsOAR-2 vs control and dsOAR-3 vs control (Figure 6.4). Interestingly, it was observed that dsOAR-1 does not significantly induce RNAi nor does it change timing of the behavioural shift (Figure 6.4). This observation from dsOAR-1 trials indirectly could serve, then, as another negative control that shows that simply feeding RNA elements does not alter the behavioural shift. At the end of group-formation trials, an equivalent number of larvae (>60%) from both control and treatments were observed to be preparing to molt from 2nd to 3rd instar, suggesting no apparent differences in transition rates from 2nd to 3rd instar between control and treatments. Overall, these results support the hypothesis that RNAi-induced downregulation of the octopamine receptor gene in 2nd instar caterpillars hastens the switch from social to the solitary behaviours.

Figure 6.4 Bar graph representing the percentage of larvae that remained solitary at the end of behavioural trials. No bar is shown for larvae treated with dsOAR-1 as none of the larvae previously treated with dsOAR-1 remained solitary. No significant differences were observed in Control vs dsOAR-1 (one-way ANOVA; Tukey's HSD, $p=0.915$), however, there were significant differences in Control vs dsOAR-2 (one-way ANOVA; Tukey's HSD, $p<0.0001$) and Control vs dsOAR-3 (one-way ANOVA; Tukey's HSD, $p<0.0001$). N= Number of replicates.

Table 6.3 RT-qPCR and behavioural assay results for individual trials for each dsRNA. Values at 48 h and 72 h represent relative transcript abundance levels calculated using double delta Ct method, Values less than or greater than 1.0 indicate a reduction or increase, respectively, in octopamine receptor transcript abundance in treated vs control caterpillars. Group formation results are provided as the total number of larvae that formed groups or remained solitary. NA = not applicable.

siRNA	Relative octopamine receptor transcript abundance		Behavioural Assays during 48 – 72 h	
	48 h	72 h	Larvae in groups	Solitary larvae
dsOAR-1				
Trial_01	0.99	0.98	5	0
Trial_02	1.25	1.65	5	0
Trial_03	0.92	2.27	4	0
MEAN $\pm$ SE	1.05 $\pm$ 0.10	1.63 $\pm$ 0.37	4.67 $\pm$ 0.33	0
dsOAR-02				
Trial_01	0.12	1.699	0	5
Trial_02	0.53	0.710	0	5
Trial_03	0.08	2.908	0	5
Trial_04	0.61	0.444	0	5
Trial_05	0.89	0.285	2	2
Trial_06	0.06	0.392	0	5
Trial_07	0.41	1.659	0	5
MEAN ($\pm$ SE)	0.39 $\pm$ 0.18	1.16 $\pm$ 0.56	0.29 $\pm$ 0.44	4.57 $\pm$ 0.65
dsOAR-03				
Trial_01	0.38	0.785	0	5
Trial_02	0.22	0.230	0	5
Trial_03	0.42	0.225	0	6
Trial_04	0.50	0.412	0	6
Trial_05	0.52	0.613	0	5
Trial_06	0.72	2.173	2	3

Trial_07	0.41	1.266	0	6
Trial_08	0.80	1.790	2	4
MEAN ($\pm$ SE)	0.50 ± 0.11	0.94 ± 0.42	0.50 ± 0.53	5.00 ± 0.62
Controls				
Trial_01	NA	NA	5	0
Trial_02	NA	NA	5	0
Trial_03	NA	NA	5	0
Trial_04	NA	NA	5	0
Trial_05	NA	NA	3	2
MEAN ($\pm$ SE)	NA	NA	4.33 ± 0.65	0.67 ± 0.67

Behaviours are complex, and often there are multiple genes that regulate a behaviour (Rittschof and Robinson 2014). Recent advances in sequencing technologies have been extensively utilized to understand genomic bases of behaviours, including social behaviours in insects (Berens et al. 2014; Shell and Rehan 2017), enabling the simultaneous identification of multiple genes associated with behaviours such as sociality by gene expression analyses. For our work on *D. arcuata*, we identified 3300 transcripts that are differentially expressed ($\log_2$ fold change ≥ 2) between instars 1 and 2 (social, group-living) vs instars 4 and 5 (solitary, territorial) (Yack et al. 2020 [see Chapter 5]). In addition to the changes in gene expression, there is a suite of morphological (e.g. head capsule color and width, body color and length) and behavioural (e.g. feeding, shelter construction, social interactions, acoustic signalling) features that undergo changes during the transition from early to late instars (Yadav and Yack 2018 [see Chapter 2]). Therefore, any gene that exhibits differential expression that correlates to behavioural transition could be ‘just another’ downstream effect of a developmental switch (Robinson et al. 2005). The main goal here is to identify the genes that are a component of the “cause” of behavioural switch, not the “consequence”. To determine the role of a particular gene in switching between behavioural phenotypes requires experimental gene function tests (reverse genomics) after identifying the gene(s) associated with a behaviour from whole genome expression analyses (forward genomics) (O’Connell and Hoffman 2011; Rubenstein and Hoffman 2015). Therefore, taking the reverse genomics approach after the identification of several sociality-related genes by forward genomics approach, knockdown experiments targeting an octopamine receptor gene using RNAi were initiated in *D. arcuata* larvae.

Octopamine has varied functions in insects that influence several physiological events, acting as a neuromodulator, neurohormone and a neurotransmitter (Farooqui 2012), and previous studies have described the roles of octopamine receptors in mediating different behaviours, including social behaviour (Verlindon et al. 2010; Lianag et al. 2012; Spindler et al. 2013; Cunningham et al. 2014; Ngai and McDowell 2017). In *D. arcuata* caterpillars, we identified an upregulation of transcripts coding for a putative octopamine receptor in group-forming early instars relative to late instars, which was further tested by conducting knockdown experiments using RNAi in the current study.

RNAi technology is increasingly being utilized in developing potential bio-pesticides against numerous insect pests, including Lepidoptera (Gong et al. 2011; Christiaens et al. 2020). For example, Gong et al. (2011, 2013) synthesized and tested insecticidal activity of dsRNA specific to the acetylcholinesterase gene in the diamondback moth, *Plutella xylostella*, providing an innovative strategy for developing biopesticides using RNAi. In another study, Rana et al. (2020) demonstrated the efficacy of using dsRNA-transgenic plant-mediated RNAi to control crop pests. Their study evaluated different families of Lepidoptera and targeted chitin synthaseA gene, opening a new avenue for designing broad-spectrum biopesticides. Also, RNAi has been applied to study a variety of other functions in Lepidoptera (reviewed in Terenius et al. 2011). However, to my knowledge there are no reported examples of using RNAi to understand molecular bases of social behaviour in Lepidoptera. The current study presents the very first example of taking an RNAi approach to exploring genetic regulation of larval grouping behaviour in a caterpillar, *D. arcuata*. The current study's findings are preliminary and should be followed

by further experiments, for example, to assess the kinetics of the RNAi response, test additional concentrations of dsRNA to find the optimal amount required to elicit social behavioural changes, and examine differences in acoustic signalling and other associated behaviours. Nonetheless, this study offers an exciting and promising approach to identifying the mechanisms of social grouping behaviour in larval Lepidoptera.

Chapter 7: Draft genome assembly and annotation of masked birch caterpillar, *Drepana arcuata* (Lepidoptera: Drepanoidea)

This chapter is an adaptation of the following publication:

Yadav, C., Smith, M.L., Ogeunremi, D. & Yack, J.E. (in review). Draft genome assembly and annotation of masked birch caterpillar, *Drepana arcuata* (Lepidoptera: Drepanoidea). *Data in Brief* (submitted August 2020)

Statement of contribution

C. Yadav collected and analyzed the data, and wrote the chapter, including the figures and tables; D. Ogunremi contributed to ONT sequencing; M. Smith contributed to the project design, molecular data collection and editing; J.E. Yack supervised the project and contributed to writing and editing.

7.1 Abstract

The masked birch caterpillar, *D. arcuata* Walker (Lepidoptera: Drepanidae), and other Drepanoidea (Lepidoptera) species are excellent organisms for investigating the function and evolution of vibratory communication and sociality in caterpillars. We present a *de novo* assembled draft genome and functional annotation for *D. arcuata*, using a combination of short and long sequencing reads generated by Illumina HiSeq X and Oxford Nanopore Technologies (ONT) MinION sequencing platforms, respectively. A total of 419,547,293 150bp paired-end Illumina and 395,890 ONT raw reads were assembled into 11,493 scaffolds spanning a genome size of 270.5Mb. The resulting *D. arcuata* genome has a GC content of 38.79%, repeat content of 8.26%, is 86.5% complete based on Benchmarking Universal Single-Copy Orthologs (BUSCO) assessment, and comprises 10,398 predicted protein-coding genes. These data represent the first genomic resources for the lepidopteran superfamily Drepanoidea. Although the order Lepidoptera comprises numerous ecologically and economically important species, assembled genomes and annotations are available for < 1% of the total species. These data can be further utilized for research on Lepidoptera genomics as well as on the function and evolution of vibratory communication and sociality in larval insects.

7.2 Introduction

This dataset presents the first draft genome assembly with functional annotation for the masked birch caterpillar, *D. arcuata* Walker (Lepidoptera: Drepanidae). Raw sequencing data used for genome assembly, and the draft genome can be accessed from NCBI Bioproject PRJNA644671 and Appendix File D7.1, respectively. Figure 7.1 presents an overview of the steps involved in assembling and annotating the draft genome. Taking a hybrid genome approach, both paired-end short reads and long sequencing reads were assembled into 11,493 scaffolds with N50 of 53.8Kb spanning 270.5Mb which represents ~90% of the estimated genome size (Gregory and Hebert 2003) (Appendix File D7.1). Appendix File D7.1 provides the sequences of scaffolds assembled. A brief summary of statistics on the draft genome and its features are provided in Table 7.1. Table 7.2 provides a summary of genome quality assessment performed by BUSCO (Benchmarking Universal Single-Copy Orthologs) (Simao et al. 2015). The genome was found to be 86.5% complete based on BUSCO, 10,398 protein coding genes were predicted (Table 7.1) and of these, >84% of the genes were functionally annotated using Blastx and InterProScan (Table 7.1) (also see Appendix Files D7.2, D7.3). Appendix File D7.2 provides sequences for putative protein coding genes and Appendix File D7.3 provides annotations done using different databases. Information on the repeat content of the assembly can be seen in Appendix Table D7.1.

Data Availability Statement: Raw DNA-seq data used in this study is at NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA644671. The assembled scaffolds (Appendix File D7.1), putative protein coding genes (Appendix File D7.2) and annotations (Appendix File D7.3) are available upon request.

Figure 7.1 Summary of methods used for draft genome assembly and annotation of *Drepana arcuata*.

Table 7.1 Characteristics of draft genome assembly, gene predictions and functional annotation of *D. arcuata*.

Genome Assembly		
Estimated genome size based on Feulgen image analysis (Mb)	303.18	
(Gregory and Hebert 2003)		
Assembled genome size (bp)	270,539,787	
Number of scaffolds	11,493	
Largest scaffold (bp)	503,976	
N50 (bp)	53,843	
N75 (bp)	26,636	
GC (%)	38.79	
Heterozygosity (%)	1.05	
Genomic Features		
Number of genes/mRNA	10,398	
Number of exons	71,785	
Number of introns	60,847	
Total genes/mRNA length (bp)	72,086,738	
Mean gene/mRNA length (bp)	6,590	
Mean exon length (bp)	13,856	
Mean intron length (bp)	845	
Mean CDS length (bp)	1,376	
% of genome covered by genes	26.6	
% of genome covered by CDS	5.6	
Mean number of exons per mRNA	7	
Repeat content (%)	8.26	
Annotation summary of predicted genes		
Database	Number	Percent (%)
InterProscan	8144	78.32
GO	5440	52.31
SwissProt	8774	84.38
Total gene	10,398	-----

Table 7.2 Summary of *D. arcuata* draft genome quality assessment done using BUSCO against Arthropoda orthologs.

Quality Assessment (BUSCO)	
Complete	86.5% (single-copy=84.5%; Duplicate=2.0%)
Fragmented	4.2%
Missing	9.3%

7.3 Experimental Design, Materials and Methods

7.3.1 *Sample collection and sequencing*

An adult male *D. arcuata* was used for DNA isolation. The specimen was obtained one day following eclosion from the pupal stage. The larva was reared from an egg laid by a wild caught female. A single individual was used for DNA extraction to simplify assembly of a single diploid genotype. A wild caught female was used since multiple rounds of inbreeding with this species is not feasible. Wings of the moth were removed and the remaining parts (head, abdomen, legs) were immediately snap-frozen in liquid nitrogen and then ground to a fine powder using a mortar and pestle. DNA extraction was done using a modified Phenol:Chloroform DNA extraction protocol (Maniatis et al. 1982). The extracted DNA was checked for purity and quantity using a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) and Qubit 4 fluorometer (ThermoFisher Scientific, Waltham, MA, USA), respectively. One µg of total DNA was submitted to Genome Quebec, McGill University, Montreal, QC, Canada, where a 2 X 150 bp shotgun paired-end library was constructed using manufacturer's instructions, followed by paired-end sequencing on an Illumina Hiseq X platform. In addition to paired-end short read sequencing, long read sequencing was performed using MinION sequencing (ONT) at Canadian Food Inspection Agency (CFIA), Ottawa, ON, Canada. Using 2 µg DNA, library preparation was performed using the 1D Ligation Sequencing kit (cat #SQK-LSK108) following manufacturer's instructions. Seventy-five µl of the prepared library was then loaded onto a MinION Flowcell R9.4 (cat # FLO-MIN106.1) according to the manufacturer's instructions and sequences were obtained for 48 hrs.

7.3.2 *Genome assembly and annotation*

A total of 460,694,612 raw reads (average quality score, $Q=36$) were obtained from Illumina HiSeqX sequencing, and 395,890 reads (quality score, $Q \geq 7$) were base-called from ONT (Nanopore sequencing) using Albacore v2.0.2. Raw reads, without any trimming (as suggested by the assembler), were then used for hybrid genome assembly using MaSuRCA v3.3.1 assembler (Zimin et al. 2013) with the default parameters. *De novo* assemblies generated using MaSuRCA were further optimized for contiguity by using AGOUTI v0.3.3 (Annotated Genome Optimization Using Transcriptome Information) (Zhang et al. 2016) using RNA- sequencing data from NCBI Bioproject PRJNA556910 (Yadav et al. 2020 [see Chapter 5]). The completeness of assembly was evaluated using BUSCO v3.0 (<https://busco.ezlab.org>) against the Arthropoda database. The draft genome assembly was annotated using WQ-Maker v2.31.9 (Thrasher et al. 2014). In the initial run, RNA-seq transcripts of *D. arcuata* accessed from DDBJ/EMBL/GenBank under accession number GIKL000000000 and protein sequences from UniProt/SwissProt protein database (accessed on May 15, 2020) were used to construct gene models. Repeat masking was also performed during this run with RepeatMasker v4.0.5 using built-in Repbase library (Bao et al. 2015). The resulting gene predictions from the initial run were used to train SNAP v2006-07-28 (Korf 2004) through a second round of WQ-Maker for gene model prediction. Next, Augustus v3.2.2 (Hoff and Stanke 2019) was trained with BUSCO using the Arthropoda ortholog database and a final round of WQ-Maker was performed with trained SNAP and Augustus for final gene model predictions.

The predicted translated protein sequences were then subjected to functional annotation using Blastp v2.6.0+ against UniProt/SwissProt database (E value cutoff of 10^{-6}), and InterProScan v5.26-65.0 for protein domain predictions (Camacho et al. 2009; Jones et al. 2014). Detailed information on repeat elements such as DNA transposons, retroelements, and total interspersed repeats was obtained on the final assembly using Repeatmasker v4.0.5 with default parameters and Arthropoda repeat database (Tarailo-Graovac et al. 2009).

7.4 Value of the Data

- This article uses both Illumina paired-end and ONT raw reads datasets to construct a draft genome for the masked birch caterpillar, *D. arcuata*, a species used in research on insect sociality and vibratory communication (Scott et al. 2010a). The study provides the first draft genome for any species of the lepidopteran superfamily Drepanoidea and addresses a knowledge gap of genome sequence within the order Lepidoptera (Triant et al. 2018).
- This dataset will be useful to entomologists interested in genomics, phylogenetics and pest control, and animal behaviourists interested in behavioural genomic studies relating to communication and sociality.
- This draft genome can be used as a reference for future genomics and evolutionary studies of the order Lepidoptera (moths and butterflies). More specifically, these data can be used to test hypotheses on the development, function, and evolution of vibratory communication and sociality in caterpillars and insects.

Chapter 8: Summary and Conclusions

“Beyond their utility as foils for comparative studies of insect societies, social caterpillars offer lessons in the marvelous complexity of insect behaviour. Social caterpillars are in many ways very different social organisms than their better known relatives, but this difference underlies an important lesson about what it means to be social, ironically pointing the way to commonalities across the sociality spectrum” (Costa, 1997)

8.1 Thesis goals

The overarching goal of my doctorate research was to explore the proximate mechanisms of sociality in larval Lepidoptera (caterpillars). Sociality is commonly observed in insects, ranging from simple group-living to complex eusocial societies. There has been much research exploring ultimate benefits and proximate mechanisms associated with sociality in eusocial insects (e.g. Miura 2004; Rehan et al. 2014; Rehan and Toth 2015). However, in simpler social systems like group-living caterpillars, although there are several studies focusing on the potential benefits associated with group-living (e.g. Costa and Pierce 1997; Ruf and Fielder 2002; Despland 2019), there is limited research exploring mechanisms of social living, such as group formation and maintenance. In this thesis, I studied two of the multiple possible mechanisms that could mediate group formation in larval Lepidoptera- vibroacoustics and genomics, using masked birch caterpillar, *D. arcuata*. These caterpillars have been previously reported to exhibit both social and solitary stages, and generate vibratory signals for territorial defense in late instars (Yack et al. 2001; Matheson 2011). There were two main objectives of this thesis- (1) to test the hypothesis that vibratory signals are used by caterpillars for recruitment of conspecifics to form groups; and (2) to

test the hypothesis that changes in gene expression are associated with larval developmental as they transition from social to solitary groups.

8.2 Summary of research chapters

Prior to conducting experiments to test these hypotheses, it was necessary to provide some baseline information on the instar stages and their behaviours. Therefore, in Chapter 2, I confirmed the number of larval instars, and for each instar, described their morphology and commented on their feeding habits and shelter construction. Five instars were confirmed based on head capsule size following Dyar's rule of geometric progression of head capsule widths in larval Lepidoptera (Dyar 1890). Although head capsule size was identified to be the most reliable feature to identify and distinguish instars from each other, other morphological features such as body length, color, and banding patterns seen on head capsules were also found to be helpful in distinguishing instars. Besides morphology, behaviours such as feeding and shelter construction that are integral to larval development were also observed. Congruent with several other larval Lepidoptera, silk shelter construction was observed throughout larval development in *D. arcuata* larvae with a change in number, types and features of shelters constructed by each instar. Additionally, changes in feeding type from skeletonization to leaf cutting was observed from early instars (I,II) to late (IV,V) instars. Interestingly, third (III) instars represented a transitional stage with both the feeding types present, changing from skeletonization to cutting as they aged. It was also noted, but not formally tested, that early instars formed communal shelters, whereas late instars remained solitary. These changes in shelter construction, feeding style and grouping may have resulted from changing needs of larvae as they grow, leading them

to switch their behaviours, as seen in other species (e.g. Despland and Hamzeh 2004). This chapter presented fundamental knowledge on the growth patterns and morphological and behavioural differences of larval instars of *D. arcuata*, which was crucial for conducting experiments related to developmental changes in social behaviour.

Whereas Chapter 2 asked ‘how many instars are there and how do we reliably distinguish between them?’, Chapter 3 asked, ‘how do instars differ in their tendency to form groups?’, and, ‘how do instars differ in how they generate vibration signals?’. I performed group formation trials for each instar to investigate the ontogenetic changes in grouping behaviour during larval development. The results indicated that instars I and II actively form groups whereas instars IV and V live solitarily. An interesting result was that third instars dispersed from communal shelters in the early part of their development to form solitary shelters in the latter part of their development, thus representing a transitional stage. In Chapter 3 I also confirmed that all five larval instars produced vibratory signals and which types of signals were produced by each instar while the caterpillars were in their natural resting states. Four signal types were identified –AS, BS, MS, and MD but in instars IV and V, no BS was reported. Also, signal rates were observed to be highest in early instars relative to late instars, regardless of their group size, and anal scrape was observed to be produced at highest signalling rates among all the signal types in all the instars. Signal rates were also observed to be highest among solitary (i.e. group size=1) individuals among all group sizes in early instars, with highest signal rates for AS. Given that early (social) instars signal more than late (solitary) instars, and, within early instars that differ in group size, those with smaller group numbers signal more, it is possible that one role of vibratory

signalling in *D. arcuata* is to recruit individuals to groups. This was tested in the next Chapter, Chapter 4. The results of Chapter 3, showing that there are distinctive changes in grouping behaviours between early and late instars, is also pertinent to Chapter 5, whereby gene expression for ‘social’ genes were compared between early and late instars.

In Chapter 4, I tested the hypothesis that vibratory signalling functioned in recruitment to groups formed by early instar *D. arcuata*. Although group-living is widespread in different caterpillars and there are several examples of species using chemical signals for recruitment (Costa 2006), to the best of my knowledge there are no examples of vibration-mediated recruitment in caterpillars. In this study, vibratory signals generated by solitary early instar resident larvae settled in silk shelters were recorded before and during recruitment of conspecific larvae. Residents were found to generate the 4 signal types- anal scrape, buzz scrape, mandible scrape and mandible drum, but anal scrape and buzz scrape were the most frequently generated signals by the resident before being joined by a conspecific, with a strong correlation to feeding and silk-laying behaviours. This suggested that the resident may be advertising feeding and shelter sites to potential recruits. Also, higher signalling rates by the resident resulted in faster joining by conspecifics. Although there could be more than one mechanism mediating group-formation in *D. arcuata* caterpillars, the hypothesis that vibratory signalling plays a role in recruitment by early instars was supported. These results provided the first evidence of vibration-mediated sociality in caterpillars.

In Chapter 5, I tested the hypothesis that differential gene expression is associated with the behavioural transition from social to solitary living in *D. arcuata* larvae. There are numerous sociogenomic studies identifying the roles that genes play in mediating social behaviours in a variety of eusocial insects (e.g., Whitfield et al. 2003; Berens et al. 2014; Dolezal and Toth 2014; Shell and Rehan 2017), indicating conservation of gene function across organisms. In fact, recent advances in sequencing technologies have made it possible to explore the genomic basis of sociality in a diversity of insects. Taking the advantage of existing knowledge on sociality-related genes identified in other insects, and sequencing technologies, I conducted *D. arcuata* larval transcriptomic analyses to identify the changes in gene expression associated with the larval transition from social to solitary behaviours. First, a de novo whole larval transcriptome was assembled and then differential expression analysis was conducted using the transcriptome as reference. This led to the identification of a large number of transcripts that differed in expression between social early and solitary late instars, including transcripts potentially coding for sociality-related gene products (e.g. bioamine receptors, cGMP- and cAMP- dependent kinases) that have been previously identified in other organisms. Also, several transcripts potentially coding for genes that could be associated with some developmental changes in larval *D. arcuata* such as body color, and feeding type were found to be differentially expressed. This study presented some interesting findings on the genomic influences on caterpillar sociality, a very first example in the insect order Lepidoptera.

Although comparative transcriptomic analyses can reveal correlations between behaviour and gene expression, taking a reverse genomic approach can help us further identify the

function that a particular gene may play in the expression of social behaviour. Therefore, in Chapter 6 I initiated gene knockdown experiments using RNA interference (RNAi) targeting one of the sociality-related genes- an octopamine receptor gene. This gene has been reported to influence sociality and related behaviours in other insects, including the solitary to gregarious phase transitions in locusts (Guo et al. 2015; Ma et al. 2015). Using chemically synthesized dsRNAs I targeted different regions of an octopamine receptor transcript sequence that was identified from transcriptomic analysis. Feeding second instar *D. arcuata* dsRNA-coated birch leaves for 48 h resulted in significant reduction of octopamine receptor transcript abundance based on RT-qPCR results. Furthermore, group-formation behaviour assessment of dsRNA fed larvae showed expedited transition to solitary state relative to the control. Findings from this preliminary investigation indicate a possible correlation of octopamine receptor gene to social to solitary behaviour transition observed in caterpillars.

Larvae belonging to the superfamily Drepanoidea vary in their social and vibratory signalling behaviour both within and between species. Therefore, this group provides a very interesting opportunity for future comparative sociogenomics research to test hypotheses on the mechanisms and evolution of social behaviour. As such, having a genome for *D. arcuata* would be a first step in facilitating future sociogenomic studies. In Chapter 7, I assembled and functionally annotated a draft genome for *D. arcuata* to a size of 270.5Mb, taking a hybrid genome approach. The draft genome represents ~90% of the estimated genome size and is 86.5% complete based on BUSCO assessment. This is the first draft genome reported for any species in the lepidopteran superfamily Drepanoidea,

adding to the genome database of Lepidoptera that lacks genomes for >99% of the species (Triant et al. 2018)

8.3 Final thoughts and future directions

My doctorate research is the first to explore proximate mechanisms of sociality in caterpillars by investigating two unprecedented mechanisms- vibroacoustics and sociogenomics. My research provided support for the hypothesis that vibratory communication plays a role in social grouping behaviour, and, that differential expression of so called ‘social’ genes may contribute to social recruitment. In the following section, I discuss some potential avenues for future research that will build upon my results.

My research confirmed that *D. arcuata* larvae generate complex vibratory signals throughout development, and provided evidence that they use vibratory communication for social recruitment. But what still remains to be understood is how the groups are maintained and how the vibratory signals function once the groups are established. I have conducted more than 50 hours of video recordings with simultaneous laser vibrometer recordings of early instar larvae while they reside in groups, and personal observations show that larvae frequently signal while in established groups. Complex vibratory events (i.e. with variation in signal rates and types) appear to correlate with different behaviours and interactions between nest mates. For example, individuals often signal prior to feeding, or working on the shelter, and individuals being approached will signal. Larvae appear to take turns feeding, and signalling appears to occur prior to and following feeding events. Therefore, some key questions that should be addressed in future investigations include the

following: How are vibrations involved in the maintenance of groups? How are vibrations involved in the coordination of activities such as shelter construction, feeding, defence? Answers to these questions can enhance our currently, fairly-limited understanding of how the tiny, pinhead sized caterpillars perform such complex tasks associated with social living.

Another key question that needs to be addressed in future research is this: How do caterpillars detect and discriminate solid-borne vibrations? To the best of my knowledge, sensory organs that detect vibrations are yet to be identified in larval insects (Yack and Yadav 2021). Antennae and various structures on or within the thorax and abdomen of caterpillars have been suggested to have a functional role in vibration reception (Dethier 1941; Hasenfuss 1992), but there is no published experimental evidence supporting this to date. Morphological and neurophysiological investigations of *D. arcuata* and *Trichoplusia ni* show that prolegs possess chordotonal sensilla as well as trichoid sensilla that touch the substrate when caterpillars are resting on a leaf, and that recordings from the proleg nerve during stimulation of these receptors show sensory activity (Rosi-Denadai 2018; Yack lab, unpublished). At present, there are no conclusive studies confirming the presence of vibration receptors in Lepidoptera (or any other insect) larvae, and how they function in receiving and discriminating signals or other vibration events. Future research on this topic can greatly enhance our knowledge of the vibratory communication ‘worlds’ of larval insects.

Sociogenomics is an emerging area of research, with increasing examples of genetic regulation of social behaviours in insects. I demonstrated differential expression of ‘social genes’ between different developmental stages of larvae that were social and solitary, and I initiated knockdown experiments using RNAi to test the role of an octopamine receptor gene in group formation. While my results supported the hypothesis that one or more genes are associated with group formation behaviour, further work is required to present solid evidence on the role(s) of the octopamine receptor gene, or other genes in mediating social behaviours. Future work could include, for example, continuing RNAi experiments with more elements, and conducting comparative studies with congener solitary species like *D. bilineata*, which is a congener of *D. arcuata* living in the same environment, but differs in that this species is solitary as a neonate. A comparison of these species could shed light on genetic mechanisms leading to profound differences in their social behaviour. Transcriptome analysis of groups vs solitary larvae within third instar *D. arcuata* would also prove interesting, as this is a transitional stage as identified by Yadav and Yack (2018 [see Chapter 2]). Answers to these questions may lead us to identifying specific genes that are associated with sociality in caterpillars, and other organisms.

My doctorate research unveiled key insights into an underappreciated but perhaps prominent sensory modality in caterpillars- vibrational communication. How these pinhead-sized neonates can exhibit complex behaviours with complex vibratory signalling is absolutely fascinating, and intriguing at the same time. My research sheds light onto the sensory world of caterpillars. I hope that my research will inspire other researchers to explore this complex vibratory landscape of larval insects. My research also probed into

genetic mechanisms that may be associated with social behaviour in larval insects. A growing number of genomic studies are revealing conserved function of sociality-related genes across taxa. My research for the very first time, provides strong evidence for this in caterpillars, by revealing sociality-related genes associated with grouping behaviour. Considering that many group-living insects are major pests, these findings could have additional benefits for pest management. Also, my research contributes genomic resources that can be utilized by researchers studying not just the behaviours, but also other aspects involving Lepidoptera biology.

Appendices

Appendix A Supplementary material for Chapter Four

Figures and files in Appendix A are supplementary materials of the published paper (<https://doi.org/10.1007/s00265-017-2280-x>), renamed for the thesis.

Figure A4.1 Waveform of the laser vibrometer recording presented in File A4.1 (see below), showing complex vibration signal repertoire of an early instar *D. arcuata* caterpillar.

File A4.1 Laser vibrometer recording (.wav file) with 4 signal types (AS,BS,MS,MD) generated by an early instar *D. arcuata*.

Available at: https://static-content.springer.com/esm/art%3A10.1007%2Fs00265-017-2280-x/MediaObjects/265_2017_2280_MOESM1_ESM.wav

File A4.2 m4v file showing AS generated by an early instar *D. arcuata* during feeding and silk shelter construction.

Available at: https://static-content.springer.com/esm/art%3A10.1007%2Fs00265-017-2280-x/MediaObjects/265_2017_2280_MOESM2_ESM.pdf

File A4.3 m4v file showing different vibratory signals generated during recruitment event, both by resident as well as the joining conspecific early instars *D. arcuata*.

Available at: https://static-content.springer.com/esm/art%3A10.1007%2Fs00265-017-2280-x/MediaObjects/265_2017_2280_MOESM4_ESM.m4v

Appendix B Supplementary material for Chapter Five

Figures and files in Appendix A are supplementary materials of the published paper (<https://doi.org/10.1371/journal.pone.0234903>), renamed for the thesis.

Figure B5.1 Length distribution of the larval *D. arcuata* transcripts assembled by Trinity. The x-axis indicates the length of transcripts (bp) and y-axis indicates the total number of transcripts for each given size range. Out of 231,348 transcripts, ~49% transcripts are above 500 bp, ~17.5% between 500 bp-1kb, and ~31.5% above 1kb in length.

Available at : <https://doi.org/10.1371/journal.pone.0234903.s001>

Figure B5.2 Representation of partitioning of *D. arcuata* transcripts into KEGG pathways. OS = Organismal systems, CP = Cellular Processes, EIP = Environmental Information Processing, GIP = Genetic Information Processing= Metabolism. The numbers on the right side of bars indicate the total number of transcripts annotated to respective KEGG pathway (labelled on the left), and x-axis shows the percentages for each pathway calculated from the total number of transcripts annotated to KEGG pathways. Metabolic processes were most represented (~26.52%), with carbohydrate, lipid and amino acid metabolism being the top pathways in this group.

Available at : <https://doi.org/10.1371/journal.pone.0234903.s002>

Figure B5.3 Histogram representing classification of clusters of orthologous groups (KOG) for the larval transcriptome. Among the 26 KOG function groups, the transcripts ‘General function prediction only’ was most represented (~16.68%) followed by ‘Signal transduction mechanisms’ (~13.35%). Percentages were calculated for each function class by dividing the total number of transcripts annotated to each class by the total number of transcripts annotated to KOG.

Available at : <https://doi.org/10.1371/journal.pone.0234903.s003>

Figure B5.4 Biological validation of RNA-seq-based DETs using RT-qPCR. Scatter plot showing transcript expression in terms of log₂-fold changes obtained from RNA-seq and RT-qPCR data for arbitrarily selected DETs. Each diamond represents a DET, N = 10. Linear regression analysis revealed high correlation between RNA-seq and RT-qPCR data ($R^2 = 0.9429$, $R = 0.9710$).

Available at : <https://doi.org/10.1371/journal.pone.0234903.s004>

Figure B5.5 Representation of partitioning of DETs into KEGG pathways. OS = Organismal systems, CP = Cellular Processes, EIP = Environmental Information Processing, GIP = Genetic Information Processing, M= Metabolism. Maximum number of DETs were identified to be associated with metabolic pathways, within which carbohydrate metabolism (~8.5%) and amino acid metabolism (~5.8%) were the top two pathways.

Available at : <https://doi.org/10.1371/journal.pone.0234903.s005>

Figure B5.6 Gene Ontology (GO) classification of differentially expressed transcripts between early and late instar *D. arcuata*. Pie chart represents the classification of transcripts in the functional category—biological process.

Available at : <https://doi.org/10.1371/journal.pone.0234903.s006>

Table B5.1 Availability of data used in the transcriptomic analysis of larval *D. arcuata*.

Available at : <https://doi.org/10.1371/journal.pone.0234903.s007>

Table B5.2 Transcript IDs and sequences of primers used for RT-qPCR validation of selected DETs in late vs early instars.

Available at : <https://doi.org/10.1371/journal.pone.0234903.s008>

Table B5.3 Summary of RNA sequencing data and transcriptome assembly for early and late instar larvae.

Available at : <https://doi.org/10.1371/journal.pone.0234903.s009>

File B5.1 Trinotate annotation report for larval *D. arcuata* transcriptome.

Available at : <https://doi.org/10.1371/journal.pone.0234903.s010>

File B5.2 Differential expression information on DETs ($\log_2FC \geq 2$; $FDR \leq 0.001$) identified between early and late instars.

Available at : <https://doi.org/10.1371/journal.pone.0234903.s011>

Appendix C Supplementary material for Chapter Six

```

T1      ccagtcgctacggcggtggccgtcttgcacgagcgttgtagtacgctacgtaatcccgta
T2      ccagtcgctacggcggtggccgttagctacgagcgttgtagtacgctacgtaatcccgta
T3      -----

T1      gctacgaacgtaacagtttgtaactagctccacgaaactgatattgtgaccgttatta
T2      gctacgaacgtaacagtttgtaactagctccacgaaactgatattgtgaccgttatta
T3      -----

T1      ttattacaatttaataatgtgagttcaataaataatattgcagatttaagaaattaact
T2      ttattacaatttaataatgtgagttcaataaataatattgcagatttaagaaattaact
T3      -----a

T1      ggaataactttt-ttgggtgacaaggaaagtgtttatactcgagctatgctgaacaccgtc
T2      ggaataactttt-ttgggtgacaaggaaagtgtttatactcgagctatgctgaacaccgtc
T3      ggalcatattatcatgagttgaattgaatgaataccgaaatgacgctaggaagggttgaa

T1      1  tacactgagattcgtttacacataaaa-aaATGAACAAGTCACATAAATGGGATTTCCTA
T2      1  tacactgagattcgtttacacataaaaaaaATGAACAAGTCACATAAATGGGATTTCCTA
T3      1  aagaaccagattcgtttacacataaaaaaaATGAACAAGTCACATAAATGGGATTTCCTA

T1      31  GAAAAGATATCAAGCAGAAGAAAAATTGTCTATAAAGGATGGATCGCGACAGTACCTCG
T2      31  GAAAAGATATCAAGCAGAAGAAAAATTGTCTATAAAGGATGGATCGCGACAGTACCTCG
T3      31  GAAAAGATATCAAGCAGAAGAAAAATTGTCTATAAAGGATGGATCGCGACAGTACCTCG

T1      91  TATGCCCTCTATA TGAAGAAATATTACCAGCGTTTATTGGCCAAATGTGTCGGCGCCGTA
T2      91  TATGCCCTCTATA TGAAGAAATATTACCAGCGTTTATTGGCCAAATGTGTCGGCGCCGTA
T3      91  TATGCCCTCTATA TGAAGAAATATTACCAGCGTTTATTGGCCAAATGTGTCGGCGCCGTA

T1      151 ACGAAGCTCAATACGCTCGCAAACACATGAAATGTAGGAAAGAAACGCAATAAAATGGGT
T2      151 ACGAAGCTCAATACGCTCGCAAACACATGAAATGTAGGAAAGAAACGCAATAAAATGGGT
T3      151 ACGAAGCTCAATACGCTCGCAAACACATGAAATGTAGGAAAGAAACGCAATAAAATGGGT

T1      211 CAAGCTTCCACTATTATCGACGGTAATTACACGTCCTCAATTATTCCGAGGTGTACGAG
T2      211 CAAGCTTCCACTATTATCGACGGTAATTACACGTCCTCAATTATTCCGAGGTGTACGAG
T3      211 CAAGCTTCCACTATTATCGACGGTAATTACACGTCCTCAATTATTCCGAGGTGTACGAG

T1      271 GTAATTGATGATGAAAAGGATGTGTGCGCCGTCGCTGATGACCCATAATACCCGAGCAGT
T2      271 GTAATTGATGATGAAAAGGATGTGTGCGCCGTCGCTGATGACCCATAATACCCGAGCAGT
T3      271 GTAATTGATGATGAAAAGGATGTGTGCGCCGTCGCTGATGACCCATAATACCCGAGCAGT

T1      331 TTTGGAATGACTCTTGCCGTGCCAGAATGGGAAGCGATATGTACAGCCATAGTGCTCACG
T2      331 TTTGGAATGACTCTTGCCGTGCCAGAATGGGAAGCGATATGTACAGCCATAGTGCTCACG
T3      331 TTTGGAATGACTCTTGCCGTGCCAGAATGGGAAGCGATATGTACAGCCATAGTGCTCACG

```

↓

dsOAR-1

OAR-F

←

OAR-F

T1 391 CTTATTATAATCTCGACGATCATAGGGAATATCCTTGTTATTCTGAGCGTGTTACAGTAC
 T2 391 CTTATTATAATCTCGACGATCATAGGGAATATCCTTGTTATTCTGAGCGTGTTACAGTAC
 T3 391 CTTATTATAATCTCGACGATCATAGGGAATATCCTTGTTATTCTGAGCGTGTTACAGTAC

T1 451 AAGCCGCTGCGAATCGTCCAGAATTTTTTCATTGTATCGCTGGCTGTCGCGGACCTGACT
 T2 451 AAGCCGCTGCGAATCGTCCAGAATTTTTTCATTGTATCGCTGGCTGTCGCGGACCTGACT
 T3 451 AAGCCGCTGCGAATCGTCCAGAATTTTTTCATTGTATCGCTGGCTGTCGCGGACCTGACT

T1 511 GTAGCAATTCTAGTGCTGCCGCTCAACGTAGCGTACTCGATACTCGGCCAGTGGGTATTCT
 T2 511 GTAGCAATTCTAGTGCTGCCGCTCAACGTAGCGTACTCGATACTCGGCCAGTGGGTATTCT
 T3 511 GTAGCAATTCTAGTGCTGCCGCTCAACGTAGCGTACTCGATACTCGGCCAGTGGGTATTCT

T1 571 GGAATTTACGTGTGCAAAATGTGGCTTACTTGCGACATAATGTGCTGCACATCGTCTATT
 T2 571 GGAATTTACGTGTGCAAAATGTGGCTTACTTGCGACATAATGTGCTGCACATCGTCTATT
 T3 571 GGAATTTACGTGTGCAAAATGTGGCTTACTTGCGACATAATGTGCTGCACATCGTCTATT

T1 631 TTAATTTATGTGCAATTGCCCTCGACAGGTATTGGGCAATTACTGACCCAATAAACTAC
 T2 631 TTAATTTATGTGCAATTGCCCTCGACAGGTATTGGGCAATTACTGACCCAATAAACTAC
 T3 631 TTAATTTATGTGCAATTGCCCTCGACAGGTATTGGGCAATTACTGACCCAATAAACTAC

T1 721 GCACAAAAAAGGACACTCGAAAGAGTGCTACTAATGATCGGCATTGTGTTGGATCCTATCG
 T2 721 GCACAAAAAAGGACACTCGAAAGAGTGCTACTAATGATCGGCATTGTGTTGGATCCTATCG
 T3 721 GCACAAAAAAGGACACTCGAAAGAGTGCTACTAATGATCGGCATTGTGTTGGATCCTATCG

T1 781 CTTGTCATTACTCGCCGCTCTACTCGGGTGAATGATTGGCCCGAAGTGTTTCATGCCG
 T2 781 CTTGTCATTACTCGCCGCTCTACTCGGGTGAATGATTGGCCCGAAGTGTTTCATGCCG
 T3 781 CTTGTCATTACTCGCCGCTCTACTCGGGTGAATGATTGGCCCGAAGTGTTTCATGCCG

T1 841 GACACTCCTTGCCGCCTTACGTCCCAACCAGGCTTCGTATATTTTCTCTTCCGGATCT
 T2 841 GACACTCCTTGCCGCCTTACGTCCCAACCAGGCTTCGTATATTTTCTCTTCCGGATCT
 T3 841 GACACTCCTTGCCGCCTTACGTCCCAACCAGGCTTCGTATATTTTCTCTTCCGGATCT

T1 901 TTCTACATACCTTTAGTTATAATGACCGTTGTGTATTTTCGAAATATATTTAGCTACGAAG
 T2 901 TTCTACATACCTTTAGTTATAATGACCGTTGTGTATTTTCGAAATATATTTAGCTACGAAG
 T3 901 TTCTACATACCTTTAGTTATAATGACCGTTGTGTATTTTCGAAATATATTTAGCTACGAAG

T1 961 AAAAGGCTAAGAGATCGCGCCAAAGCGACCAAAATCAGTACTATATCTAGCGGGCAGAAAT
 T2 961 AAAAGGCTAAGAGATCGCGCCAAAGCGACCAAAATCAGTACTATATCTAGCGGGCAGAAAT
 T3 961 AAAAGGCTAAGAGATCGCGCCAAAGCGACCAAAATCAGTACTATATCTAGCGGGCAGAAAT

T1 1021 AAATACACAGTGAAAGACAATGATCACCACGATCAAGACTCAGTGAGCTCTGAGGCAAAAT
 T2 1021 AAATACACAGTGAAAGACAATGATCACCACGATCAAGACTCAGTGAGCTCTGAGGCAAAAT
 T3 1021 AAATACACAGTGAAAGACAATGATCACCACGATCAAGACTCAGTGAGCTCTGAGGCAAAAT

T1 1081 CACAATGAACATCAAGGCGTCACGCGGCTTGTTTCAAACGAGAGTGAGAAAAAGAGAGA
 T2 1081 CACAATGAACATCAAGGCGTCACGCGGCTTGTTTCAAACGAGAGTGAGAAAAAGAGAGA
 T3 1081 CACAATGAACATCAAGGCGTCACGCGGCTTGTTTCAAACGAGAGTGAGAAAAAGAGAGA


```

T2      agatacgggtgttactacagtaattaagttatgaaatatattctaaggtaattgtttcgtc
T3      agatacgggtgttactacagtaattaagttatgaaatatattctaaggtaattgtttcgtc

T1      gataaatcgatacgaatctaaatgtatgaatgtaacttggtgattattaaggatattgtga
T2      gataaatcgatacgaatctaaatgtatgaatgtaacttggtgattattaaggatattgtga
T3      gataaatcgatacgaatctaaatgtatgaatgtaacttggtgattattaaggatattgtga

T1      attcatgtggtttttattaaactatggaactcgtgagtagttaaatactagtagaagt
T2      attcatgtggtttttattaaactagcagccgtggtggccgaatggttcgagcagttgcctg
T3      attcatgtggtttttattaaactagcagccgtggtggccgaatggttcgagcagttgcctg

T1      gcattgcacgtagtgggaaatctataaattcctaattcaatcgaattataatgtacacg
T2      cgacgcaaaggtcg---caagttcgaatcccgactgtaccaatgagtttttctga--act
T3      cgacgcaaaggtcg---caagttcgaatcccgactgtaccaatgagtttttctga--act

T1      taataattgtaatacaacgaatattacgatctaataataatattaatattatcgttgtaa
T2      taagtacag-----
T3      taagtacag-----

T1      tacatatctggtccgttgtaaatatggatggaaataacaataatttatattattcaaaag
T2      -----
T3      -----

T1      taattgtttattagctaatactgagataaattaaaattataggtagtcattttcataagt
T2      -----
T3      -----

T1      ttaattattatccctttgaaataatttgagcccttattttaaggggatattagacatta
T2      -----
T3      -----

T1      taccagat
T2      -----
T3      -----

```

Figure C6.1 ClustalOmega generated alignment of OAR transcript isoforms (T1, T2, T3) retrieved from *D. arcuata* transcriptome GIKL00000000. Boxshading was generated by BoxShade server (https://embnet.vital-it.ch/software/BOX_form.html). Identical sequences are represented by white letters against black background, and dashes between nucleotides represent gaps that were introduced by ClustalOmega to maximize alignment. Putative coding sequences are represented by uppercase letters, and the numbering of the sequences begins at the beginning (downward blue arrow) and ends at the end of putative coding sequence (upward blue arrow). Locations of 3 dsRNAs are indicated by green boxes around the sequences, and location of primer sequences used for RT-qPCR are indicated by forward and backward arrows(red).

Appendix D Supplementary material for Chapter Seven

Figures and files in Appendix D are supplementary materials of the manuscript under review by Data in Brief, renamed for the thesis.

Table D7.1 Summary of repeat content in *D. arcuata* genome generated by RepeatMasker using the arthropod repeat database.

Total sequences : 11,493			
Total length:	270,539,787		
GC level:	38.79%		
Bases masked:	22,335,690 (8.28%)		
	Number of elements*	Length occupied (bp)	Percentage of sequence
Retroelements	68,009	13,853,897	5.12
SINEs:	15,042	1,371,415	0.51
LINEs:	51,054	12,229,221	4.52
LTR elements:	1913	253,361	0.09
DNA transposons	24,608	2,399,250	0.89
Rolling-circles	50,136	6,037,860	2.23
Unclassified:	1	88	0.00
Total interspersed repeats:		16,253,235	6.01
Small RNA:	61	44,595	0.02
Satellites:	0	0	0.00
Simple repeats:	0	0	0.00
Low complexity:	0	0	0.00

* most repeats fragmented by insertions or deletions have been counted as one element

File D7.1 Fasta sequences of scaffolds assembled by MaSuRCA and AGOUTI.
(available upon request)

File D7.2 Fasta sequences of *D. arcuata* putative proteins identified by WQ-MAKER.
(available upon request)

File D7.3 Structural and functional annotations of *D. arcuata* putative genes identified by WQ-MAKER.
(available upon request)

References

- Abarca, M., Boege, K., & Zaldívar-Riverón, A. (2014). Shelter-building behaviour and natural history of two pyralid caterpillars feeding on *Piper stipulaceum*. *Journal of Insect Science*, 14, 39. <https://doi.org/10.1093/jis/14.1.39>
- Alcock, J. (2005). *Animal Behaviour: An Evolutionary Approach* (8th ed.). Sinauer Associates Press.
- Ament, S.A., Wang, Y., Chen, C.C., Blatti, C.A., Hong, F., Liang, Z.S., et al. (2012). The transcription factor Ultraspiracle influences honey bee social behaviour and behaviour-related gene expression. *PLoS Genetics*, 8, Article e1002596. <https://doi.org/10.1371/journal.pgen.1002596>
- Anstey, L.J., Quiring, D.T., & Ostaff, D.P. (2002). Seasonal changes in intra-tree distribution of immature balsam fir sawfly (Hymenoptera: Diprionidae). *Canadian Entomologist*, 134, 529–538. <https://doi.org/10.4039/Ent134529-4>
- Badisco, L., Ott, S.R., Rogers, S.M., Matheson, T., Knapen, D., Vergauwen, L., et al. (2011). Microarray-based transcriptomic analysis of differences between long term gregarious and solitary locusts. *PLoS ONE*, 6, Article e28110. <https://doi.org/10.1371/journal.pone.0028110>
- Bao, W., Kojima, K.K., & Kohany, O. (2015). Repbase Update, a database of repetitive elements in eukaryotic genomes. *Mobile DNA*, 6, 11. <https://doi.org/10.1186/s13100-015-0041-9>
- Barrionuevo, M., & San Blas, G. (2016). Redescription of immature stages of the soybean looper (Lepidoptera: Noctuidae: Plusiinae). *Canadian Entomologist*, 148, 247–259. <https://doi.org/10.4039/tce.2015.62>
- Batra, S. (1966). Nests and social behaviour of halictine bees of India (Hymenoptera: Halictidae). *Indian Journal of Entomology*, 28, 375–393.
- Ben-Shahar, Y., Leung, H.T., Pak, W.L., Sokolowski, M.B., & Robinson, G.E. (2003). cGMP dependent changes in phototaxis: a possible role for the foraging gene in honeybee division of labour. *Journal of Experimental Biology*, 206, 2507–2515.

- Ben-Shahar, Y., Robichon, A., Sokolowski, M.B., & Robinson, G.E. (2002). Influence of gene action across different time scales on behaviour. *Science*, 296(5568), 741–744. <https://doi.org/10.1126/science.1069911>
- Berens, A.J., Hunt, J.H., & Toth, A.L. (2014). Comparative transcriptomics of convergent evolution: different genes but conserved pathways underlie caste phenotypes across lineages of eusocial insects. *Molecular Biology and Evolution*, 32(3), 690–703. <https://doi.org/10.1093/molbev/msu330>
- Bernays, E.A., & Janzen, D.H. (1988). Saturniid and sphingid caterpillars: two ways to eat leaves. *Ecology*, 69, 1153–1160. <https://www.jstor.org/stable/1941269>
- Beutenmuller, W. (1898). Bombycine moths of vicinity of N.Y. *Bulletin of the American Museum of Natural History*, 17, 388–389.
- Bloch, G., Toma, D.P., & Robinson, G.E. (2001). Behavioural rhythmicity, age, division of labor and period expression in the honeybee brain. *Journal of Biological Rhythms*, 16, 444–456. <https://doi.org/10.1177/074873001129002123>
- Blumstein, D.T., Daniel, J.C., & Evans, C.S. (2010). JWatcher V1.0. University of California, Los Angeles and Macquarie University, Sydney.
- Boettcher, M., & McManus, M.T. (2015). Choosing the right tool for the job: RNAi, TALEN, or CRISPR. *Molecular Cell*, 58(4), 575–585. <https://doi.org/10.1016/j.molcel.2015.04.028>
- Bolger, A.M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina Sequence Data. *Bioinformatics*, 30, 2114–2120. <https://doi.org/10.1093/bioinformatics/btu170>
- de Bono, M., & Bargmann, C. (1998). Natural variation in a neuropeptide Y receptor homolog modifies social behaviour and food response in *C. elegans*. *Cell*, 94, 679–689. [https://doi.org/10.1016/S0092-8674\(00\)81609-8](https://doi.org/10.1016/S0092-8674(00)81609-8)
- Borzoui, E., Bandani, A.R., Goldansaz, S.H., & Talaei-Hassanlouei, R. (2018). Dietary protein and carbohydrate levels affect performance and digestive physiology of *Plodia interpunctella*. *Journal of Economic Entomology*, 111(2), 942–949. <https://doi.org/10.1093/jee/tox360>

- Bowen, J.L., Mahony, S.J., Mason, A.C., & Yack, J.E. (2008). Vibration-mediated territoriality in the warty birch caterpillar *Drepana bilineata*. *Physiological Entomology*, 33, 238–250.
<https://doi.org/10.1111/j.1365-3032.2008.00627.x>
- Bradbury, J.W., & Vehrencamp, S.L. (2011). *Principles of Animal Communication*. (2nd ed.). Sinauer Associates, Sunderland.
- Brodersen, P., & Voinnet, O. (2009). Revisiting the principles of microRNA target recognition and mode of action. *Nature Reviews Molecular Cell Biology*, 10(2), 141–148). <https://doi.org/10.1038/nrm2619>
- Bryant, D.M., Johnson, K., Di Tommaso, T., Tickle, T., Couger, M.B., Payzin-Dogru D., et al. (2017). A tissue-mapped axolotl de novo transcriptome enables identification of limb regeneration factors. *Cell Reports*, 18(3), 762–776.
<https://doi.org/10.1016/j.celrep.2016.12.063>
- Bryant, S.R., Thomas, C.D. & Bale, J.S. (2000). Thermal ecology of gregarious and solitary nettle-feeding nymphalid butterfly larvae. *Oecologia*, 122, 1–10.
<https://doi.org/10.1007/PL00008825>
- Bryner, R. (1999). Drepanidae - Drépanidés. In *Les Papillons et leur Biotopes: Espèces; Danger qui les menacent; Protection. Suisse et regions limitrophe* (Vol. 2, pp. 447–476) (ed. L. S. p. I. P. d. I. Nature) Switzerland: Pro Natura.
- Caine, N.G., Addington, R.L., & Windfelder, T.L. (1995). Factors affecting the rates of food calls given by red-bellied tamarins. *Animal Behaviour*, 50, 53–60.
<https://doi.org/10.1006/anbe.1995.0220>
- Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., et al. (2009). BLAST+: Architecture and applications. *BMC Bioinformatics*, 10, 421.
<https://doi.org/10.1186/1471-2105-10-421>
- Castellanos, I., & Barbosa, P. (2006). Evaluation of predation risk by a caterpillar using substrate-borne vibrations. *Animal Behaviour*, 72, 461–469.
<https://doi.org/10.1016/j.anbehav.2006.02.005>
- Cazado, L.E., Van Nieuwenhove, G.A., O'Brien, C.W., Gastaminza, G.A. & Murúa, M.G. (2014). Determination of number of instars of *Rhyssomatus subtilis*

- (Coleoptera: Curculionidae) based on head capsule widths. *Florida Entomologist*, 97, 639–643. <https://doi.org/10.1653/024.097.0241>
- Chen, S., Yang, P., Jiang, F., Wei, Y., Ma, Z., & Kang, Le. (2010). De novo analysis of transcriptome dynamics in the migratory locust during the development of phase traits. *PLoS ONE*, 5, Article e15633. <https://doi.org/10.1371/journal.pone.0015633>
- Choe, J. C., & Crespi, B. J. (1997). *The Evolution of Social Behaviour in Insects and Arachnids*. Cambridge University Press, Cambridge. <https://doi.org/10.1017/CBO9780511721953>
- Choi, M.Y., & Vander Meer, R.K. (2019). Phenotypic effects of PBAN RNAi using oral delivery of dsRNA to corn earworm (Lepidoptera: Noctuidae) and tobacco budworm larvae. *Journal of Economic Entomology*, 112(1), 434–439. <https://doi.org/10.1093/jee/toy356>
- Christiaens, O., Whyard, S., Véléz, A.M., & Smagghe, G. (2020). Double-stranded RNA technology to control insect pests: current status and challenges. *Frontiers in Plant Science*, 11, 451. <https://doi.org/10.3389/fpls.2020.00451>
- Clay, Z., Smith, C.L., & Blumstein, D.T. (2012). Food-associated vocalizations in mammals and birds: what do these calls really mean? *Animal Behaviour*, 83, 323–330. <https://doi.org/10.1016/j.anbehav.2011.12.008>
- Cocroft, R.B. (1996). Insect vibrational defence signals. *Nature*, 382, 679–680. <https://doi.org/10.1038/382679a0>
- Cocroft, R.B. (2001). Vibrational communication and the ecology of group-living, herbivorous insects. *American Zoologist*, 41, 1215–1221. <https://doi.org/10.1093/icb/41.5.1215>
- Cocroft, R.B. (2005). Vibrational communication facilitates cooperative foraging in a phloem-feeding insect. *Proceedings of Royal Society London B: Biological Sciences*, 272, 1023–1029. <https://doi.org/10.1098/rspb.2004.3041>
- Cocroft, R.B., & Hamel, J.A. (2010). Vibrational communication in the “other insect societies”: A diversity of ecology, signals and signal functions. In C.E. O’Connell-Rodwell (Ed.), *The Use of Vibrations in Communication: Properties,*

- Mechanisms and Function Across Taxa* (pp. 47-68). Transworld Research Network, Kerala.
- Cocroft, R.B., & Rodríguez, R.L. (2005). The behavioural ecology of insect vibrational communication. *Bioscience*, 55, 323–334.
[https://doi.org/10.1641/0006-3568\(2005\)055\[0323:TBEQIV\]2.0.CO;2](https://doi.org/10.1641/0006-3568(2005)055[0323:TBEQIV]2.0.CO;2)
- Costa, J.T. (1997). Caterpillars as social insects. *American Scientist*, 85(2), 150–159.
- Costa, J.T. (2006). *The Other Insect Societies*. The Belknap Press of Harvard University Press, Cambridge.
- Costa, J.T. (2010). Social evolution in ‘other’ insects and arachnids. In M.D. Breed & J. Moore (Eds.), *Encyclopedia of Animal Behaviour* (Vol. 3, pp. 231–241). Academic Press, Oxford.
- Costa, J.T. (2018). The other insect societies: overview and new directions. *Current Opinions in Insect Science*, 28, 40–49. <https://doi.org/10.1016/j.cois.2018.04.008>
- Costa, J.F., Cosio, W., & Gianoli, E. (2007). Group size in a gregarious tortoise beetle: patterns of oviposition vs. larval behaviour. *Entomologia Experimentalis et Applicata*, 125, 165–169.
- Costa, J.T., & Fitzgerald, T.D. (2005). Social terminology revisited: where are we ten years later? *Annales Zoologici Fennici*, 42, 559-564.
- Costa, J.T., Fitzgerald, T.D., Pescador-Rubio, A., Mays, J., & Janzen, D.H. (2004). Group foraging behaviour of the larvae of the Neotropical processionary weevil *Phelypera distigma* (Boheman) (Coleoptera: Curculionidae: Hyperinae). *Ethology*, 110, 515–530.
- Costa, J.T., & Pierce, N.E. (1997). Social evolution in the Lepidoptera: ecological context and communication in larval societies. In J.C. Choe & B.J. Crespi (Eds.), *The Evolution of Social Behaviour in Insects and Arachnids* (pp. 407–422). Cambridge University Press, Cambridge.
- Cohen, R.W., Mahoney, D.A., & Can, H.D. (2002). Possible regulation of feeding behaviour in cockroach nymphs by the neurotransmitter octopamine. *Journal of Insect Behaviour*, 15(1), 37–50. <https://doi.org/10.1023/A:1014428011892>
- Cokl, A., & Virant-Doberlet, M. (2003). Communication with substrate-borne signals in small plant - dwelling insects. *Annual Review of Entomology*, 48, 29-50.

<https://doi.org/10.1146/annurev.ento.48.091801.112605>

Colasurdo, N., & Despland, E. (2005). Social cues and following behaviour in the forest tent caterpillar. *Journal of Insect Behaviour*, 18, 77–87.

<https://doi.org/10.1007/s10905-005-9348-6>

Cremer, S., Pull, C.D., & Fürst, M.A. (2018). Social Immunity: emergence and evolution of colony-level disease protection. *Annual Review of Entomology*, 63, 105–123.

<https://doi.org/10.1146/annurev-ento-020117-043110>

Cui, L., Rui, C., Yang, D., Wang, Z., & Yuan, H. (2017a). De novo transcriptome and expression profile analyses of the Asian corn borer (*Ostrinia furnacalis*) reveals relevant flubendiamide response genes. *BMC Genomics*, 18, 20.

<https://doi.org/10.1186/s12864-016-3431-6>

Cui, M., Hu, P., Wang, T., Tao, J., & Zong, S. (2017b). Differential transcriptome analysis reveals genes related to cold tolerance in sea buckthorn carpenter moth, *Eogystia hippophaecolus*. *PLoS ONE*, 12(11), Article e0187105.

<https://doi.org/10.1371/journal.pone.0187105>

Cunningham, C.B., Douthit, M.K., & Moore, A.J. (2014). Octopaminergic gene expression and flexible social behaviour in the subsocial burying beetle *Nicrophorus vespilloides*. *Insect Molecular Biology*, 23(3), 391–404.

<https://doi.org/10.1111/imb.12090>

Delbac, L., Lecharpentier, P., & Thiery, D. (2010). Larval instars determination for the European Grapevine Moth (Lepidoptera: Tortricidae) based on the frequency distribution of head-capsule widths. *Crop Protection*, 29, 623–630.

<https://doi.org/10.1016/j.cropro.2010.01.009>

Denno, R.F., & Benrey, B. (1997). Aggregation facilitates larval growth in the neotropical nymphalid butterfly *Chlosyne janais*. *Ecological Entomology*, 22, 133–141.

Despland, E. (2019). Caterpillars cooperate to overcome plant glandular trichome defenses. *Frontiers in Ecology and Evolution*, 7, 1–9.

<https://doi.org/10.3389/fevo.2019.00232>

- Despland, E., & Hamzeh, S. (2004). Ontogenetic changes in social behaviour in the forest tent caterpillar, *Malacosoma disstria*. *Behavioural Ecology and Sociobiology*, 56, 177–184. <https://doi.org/10.1007/s00265-004-0767-8>
- Despland, E., & Le Huu, A. (2007). Pros and cons of group living in the forest tent caterpillar: separating the roles of silk and of grouping. *Entomologia Experimentalis et Applicata*, 122, 181–189. <https://doi.org/10.1111/j.1570-7458.2006.00512.x>
- Despland, E., & Santacruz-Endara, P. (2016). Silk drives aggregation and following in the neotropical Ithomiine caterpillar *Mechanitis menapis*. *Physiological Entomology*, 41, 274–280. <https://doi.org/10.1111/phen.12153>
- Dethier, V.G. (1941). The function of the antennal receptors in lepidopterous larvae. *Biological Bulletin*, 80, 403–414.
- DeVries, P.J. (1990). Enhancement of symbioses between butterfly caterpillars and ants by vibrational communication. *Science*, 248, 1104–1106. <https://doi.org/10.1126/science.248.4959.1104>
- Dew, R.M., Tierney, S.M., & Schwarz, M.P. (2016). Social evolution and casteless societies: needs for new terminology and a new evolutionary focus. *Insectes Sociaux*, 63, 5–14. <https://doi.org/10.1007/s00040-015-0435-1>
- Di Bitetti, M.S. (2003). Food-associated calls of tufted capuchin monkeys (*Cebus apella nigratus*) are functionally referential signals. *Behaviour*, 140, 565–592. <https://psycnet.apa.org/doi/10.1163/156853903322149441>
- Di Bitetti, M.S. (2005). Food-associated calls and audience effects in tufted capuchin monkeys, *Cebus apella nigratus*. *Animal Behaviour*, 69, 911–919. <https://doi.org/10.1016/j.anbehav.2004.05.021>
- Dierick, H.A. (2008). Fly fighting: octopamine modulates aggression. *Current Biology*, 18(4), 161–163. <https://doi.org/10.1016/j.cub.2007.12.026>
- Dolezal, A.G., & Toth, A.L. (2014). Honey bee sociogenomics: a genome-scale perspective on bee social behaviour and health. *Apidologie*, 45, 375–395. <https://doi.org/10.1007/s13592-013-0251-4>

- Dussutour, A., Nicolis, S.C., Despland, E., & Simpson, S.J. (2008). Individual differences influence collective behaviour in social caterpillars. *Animal Behaviour*, 76, 5–16. <https://doi.org/10.1016/j.anbehav.2007.12.009>
- Dyar, H.G. (1890). The number of molts of lepidopterous larvae. *Psyche* 5, 420–422.
- Dyar, H.G. (1895). Notes on drepanid larvae. *Journal of New York Entomological Society*, 3, 66–69.
- Edgar, R., Domrachev, M., & Lash, A.E. (2002). Gene expression omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Research*, 30, 207–210. <https://doi.org/10.1093/nar/30.1.207>
- Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., & Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*, 411(6836), 494–498. <https://doi.org/10.1038/35078107>
- Elgar, M.A. (1986). House sparrows establish flocks by giving chirrup calls if the resources are divisible. *Animal Behaviour*, 34, 169–174.
- Endo, J., Takanashi, T., Mukai, H., & Numata, H. (2019). Egg-cracking vibration as a cue for stink bug siblings to synchronize hatching. *Current Biology*, 29, 143–148. <https://doi.org/10.1016/j.cub.2018.11.024>
- Farooqui, T. (2012). Review of octopamine in insect nervous systems. *Open Access Insect Physiology*, 4, 1–17. <https://doi.org/10.2147/oaip.s20911>
- Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., & Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, 391(6669), 806–811. <https://doi.org/10.1038/35888>
- Fitzgerald, T.D. (1993). Sociality in Caterpillars. In N.E. Stamp & T.M. Casey (Eds.), *Caterpillars: Ecological and Evolutionary Constraints on Foraging* (pp. 372–403). New York: Chapman and Hall.
- Fitzgerald, T. D. (2003). Role of trail pheromone in foraging and processionary behaviour of pine processionary caterpillars *Thaumetopoea pityocampa*. *Journal of Chemical Ecology*, 29, 513–532. <https://doi.org/10.1023/A:1022875102682>
- Fitzgerald, T.D., & Clark, K. (1994). Analysis of leaf-rolling behaviour of *Caloptilia serotinella* (Lepidoptera: Gracillariidae). *Journal of Insect Behaviour*, 7, 859–872.

- Fitzgerald, T.D., Clark, K., Vanderpool, R., & Phillips, C. (1991). Leaf shelter- building caterpillars harness forces generated by axial retraction of stretched and wetted silk. *Journal of Insect Behaviour*, 4, 21–32. <https://doi.org/10.1007/BF01092548>
- Fitzgerald, T.D., & Costa, J.T. (1999). Collective behaviour in social caterpillars. In C. Detrain, J.L. Deneubourg, & J.M. Pasteels (Eds.), *Information Processing in Social Insects* (pp. 379-400). Birkhäuser, Basel.
- Fitzgerald, T.D., & Pescador-Rubio, A. (2002). The role of tactile and chemical stimuli in the formation and maintenance of the processions of the social caterpillar *Hylesia lineata* (Lepidoptera: Saturniidae). *Journal of Insect Behaviour*, 15, 659–674. <https://doi.org/10.1023/A:1020795806582>
- Fitzgerald, T.D., & Peterson, S.C. (1988). Cooperative foraging and communication in social caterpillars. *Bioscience*, 38, 20–25.
- Fitzpatrick, M.J., Ben-Shahar, Y., Smid, H.M., Vet, L.E.M., Robinson, G.E., & Sokolowski, M.B. (2005). Candidate genes for behavioural ecology. *Trends in Ecology and Evolution*, 20, 96–104. <https://doi.org/10.1016/j.tree.2004.11.017>
- Fletcher, L.E. (2007). Vibrational signals in a gregarious sawfly larva (*Perga affinis*): Group coordination or competitive signalling? *Behavioural Ecology and Sociobiology*, 61, 1809-1821. <https://doi.org/10.1007/s00265-007-0414-2>
- Fletcher, L.E. (2008). Cooperative signalling as a potential mechanism for cohesion in a gregarious sawfly larva, *Perga affinis*. *Behavioural Ecology and Sociobiology*, 62, 1127-1138. <https://doi.org/10.1007/s00265-007-0541-9>
- Fletcher, L.E. (2009). Examining potential benefits of group living in a sawfly larva, *Perga affinis*. *Behavioural Ecology*, 20 (3), 657-664. <https://doi.org/10.1093/beheco/arp048>
- Fletcher, L.E., Yack, J.E., Fitzgerald, T.D., & Hoy, R.R. (2006). Vibrational communication in the cherry leaf roller caterpillar *Caloptilia serotinella*. *Journal of Insect Behaviour*, 19, 1-18. <https://doi.org/10.1007/s10905-005-9007-y>
- Fordyce, J.A. (2006). Between-clutch interactions affect a benefit of group feeding for pipevine swallowtail larvae. *Ecological Entomology*, 31, 75-83. <https://doi.org/10.1111/j.0307-6946.2006.00760.x>

- Fordyce, J.A., & Agrawal, A. (2001). The role of plant trichomes and caterpillar group size on growth and defence of the pipevine swallowtail *Battus philenor*. *Journal of Animal Ecology*, 70, 997–1005.
<https://doi.org/10.1046/j.0021-8790.2001.00568.x>
- Fussnecker, B.L., Smith, B.H., & Mustard, J.A. (2006). Octopamine and tyramine influence the behavioural profile of locomotor activity in the honey bee (*Apis mellifera*). *Journal of Insect Physiology*, 52(10), 1083–1092.
<https://doi.org/10.1016/j.jinsphys.2006.07.008>
- Gaines, J.C., & Campbell, F.L. (1935). Dyar's rule as related to the number of instars of the corn ear worm, *Heliothis obsoleta* (Fab.), collected in the field. *Annals of the Entomological Society of America*, 28, 445–461.
- Gilbert, C. (1994). Form and function of stemmata in larvae of holometabolous insects. *Annual Review of Entomology*, 39, 323–349.
- Gish, M., Dafni, A., & Inbar, M. (2012). Young aphids avoid erroneous dropping when evading mammalian herbivores by combining input from two sensory modalities. *PLoS One*, 7(4), Article e32706. [doi:10.1371/journal.pone.0032706](https://doi.org/10.1371/journal.pone.0032706)
- Goldstein, P.Z. (2017). Diversity and significance of Lepidoptera: a phylogenetic perspective. In R.G. Footitt & P.H. Adler (Eds.), *Insect Biodiversity: Science and Society* (2nd ed., Vol. 1, pp. 463–495). Oxford: J Wiley & Sons.
<https://doi.org/10.1002/9781118945568.ch13>
- Gong, L., Chen, Y., Hu, Z., & Hu, M. (2013). Testing insecticidal activity of novel chemically synthesized siRNA against *Plutella xylostella* under laboratory and field conditions. *PLoS ONE*, 8(5), 1–8.
<https://doi.org/10.1371/journal.pone.0062990>
- Gong, L., Yang, X., Zhang, B., Zhong, G., & Hu, M. (2011). Silencing of Rieske iron sulfur protein using chemically synthesised siRNA as a potential biopesticide against *Plutella xylostella*. *Pest Management Science*, 67(5), 514–520.
<https://doi.org/10.1002/ps.2086>
- Gospocic, J., Shields, E.J., Glastad, K.M., Lin, Y., Penick, C.A., Yan, H., et al. (2017). The neuropeptide corazonin controls social behaviour and caste identity in ants. *Cell*, 170, 748–759. <https://doi.org/10.1016/j.cell.2017.07.014>

- Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., et al. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology*, 29, 644–652.
<https://doi.org/10.1038/nbt.1883>
- Gregory, T.R., & Hebert, P.D.N. (2003). Genome size variation in lepidopteran insects, *Canadian Journal of Zoology*, 81, 1399-1405. <https://doi.org/10.1139/z03-126>
- Gros-Louis, J. (2004). Responses of white-faced capuchins (*Cebus capucinus*) to naturalistic and experimentally presented food associated calls. *Journal of Comparative Psychology*, 118(4), 396–402.
<https://psycnet.apa.org/doi/10.1037/0735-7036.118.4.396>
- Guedes, R.N.C., Matheson, S.M., Frei, B., Smith, M.L., & Yack, J.E. (2012). Vibration detection and discrimination in the masked birch caterpillar (*Drepana arcuata*). *Journal of Comparative Physiology A*, 198, 325–335.
<https://doi.org/10.1007/s00359-012-0711-8>
- Guo, W., Wang, X.H., Ma, Z.Y., Xue, L.A., Han, J.Y., Yu, D., et al. (2011). CSP and Takeout genes modulate the switch between attraction and repulsion during behavioural phase change in the migratory locust. *PLoS Genetics*, 7, Article e1001291. <https://doi.org/10.1371/journal.pgen.1001291>
- Guo, X, Ma, Z., & Kang, L. (2015). Two dopamine receptors play different roles in phase change of the migratory locust. *Frontiers in Behavioural Neuroscience*, 9, 1–13. <https://doi.org/10.3389/fnbeh.2015.00080>
- Handfield, L. (1999). *Le guide des papillons du Quebec*. Broquet inc., Quebec, Canada.
- Hasenfuss, I. (1992). Morphology, evolution, and taxonomic importance of supposed web- vibration receptors in the larvae of butterflies (Lepidoptera: Pyraloidea and Gelechioidea). *Entomologica Generalis*, 18, 43-54.
- Hewlett, S.E., Delahunt Smoleniec, J.D., Wareham, D.M., Pyne, T.M., & Barron, A.B. (2018). Biogenic amine modulation of honey bee sociability and nestmate affiliation. *PLoS ONE*, 13(10), Article e0205686.
<https://doi.org/10.1371/journal.pone.0205686>
- Hill, D.S. (1983). *Agricultural Insect Pests of the Tropics and Their Control* (pp. 551). London: Cambridge University Press.

- Hill, P.S.M. (2008). *Vibration Communication in Animals*. Harvard University Press, London.
- Hochuli, D.F. (2001). Insect herbivory and ontogeny: how do growth and development influence feeding behaviour, morphology and host use? *Austral Ecology*, 26, 563–570. <https://doi.org/10.1046/j.1442-9993.2001.01135.x>
- Hoff, K.J., & Stanke, M. (2019). Predicting genes in single genomes with Augustus. *Current Protocols in Bioinformatics*, 65(1), Article e57. <https://doi.org/10.1002/cpbi.57>
- Hograefe, T. (1984). Subtrat-stridulation bei den koloniebildenden Blattwespenlarven von *Hemichroa crocea* (Geoff.) (Hymenoptera: Tenthredinidae). *Zoologischer Anzeiger*, 213, 234–241.
- Hrncir, M., & Barth, F.G. (2014). Vibrational communication in stingless bees (Meliponini): the challenge of interpreting the signals. In R.B. Cocroft, M. Gogala, P.S.M Hill, & A. Wessel (Eds.), *Studying Vibrational Communication* (pp. 349–374). Springer, New York. https://doi.org/10.1007/978-3-662-43607-3_18
- Hunt, G.J., & Gadau, J.R. (2016). Editorial: advances in genomics and epigenomics of social insects. *Frontiers in Genetics*, 7, 1–2. <https://doi.org/10.3389/fgene.2016.00199>
- Hunt, J.H., & Richard, F.J. (2013). Intracolony vibroacoustic communication in social insects. *Insectes Sociaux*, 60, 403–417. <https://doi.org/10.1007/s00040-013-0311-9>
- Hunter, A.F. (2000). Gregariousness and repellent defences in the survival of phytophagous insects. *Oikos*, 91, 213. <https://doi.org/10.1034/j.1600-0706.2000.910202.x>
- Hunter, M.D., & Willmer, P.G. (1989). The potential for interspecific competition between two abundant defoliators on oak: leaf damage and habitat quality. *Ecological Entomology*, 14, 267–277. <https://doi.org/10.1111/j.1365-2311.1989.tb00956.x>

- Ishay, J., Motro, A., Gitter, S., & Brown, M.B. (1974). Rhythms in acoustical communication by the oriental hornet, *Vespa orientalis*. *Animal Behaviour*, 22, 741-744. [https://doi.org/10.1016/S0003-3472\(74\)80026-6](https://doi.org/10.1016/S0003-3472(74)80026-6)
- Jandt, J.M., & Toth, A.L. (2015). Physiological and genomic mechanisms of social organization in wasps (family: Vespidae). *Advances in Insect Physiology*, 48, 95-130. <https://doi.org/10.1016/bs.aiip.2015.01.003>
- Joga, M.R., Zotti, M.J., Smagghe, G., & Christiaens, O. (2016). RNAi efficiency, systemic properties, and novel delivery methods for pest insect control: what we know so far. *Frontiers in Physiology*, 7, 553. <https://doi.org/10.3389/fphys.2016.00553>
- Jones, P., Binns, D., Chang, H.Y., Fraser, M., Li, W., McAnulla, C., et al. (2014). InterProScan 5: genome-scale protein function classification. *Bioinformatics*, 30(9), 1236–1240. <https://doi.org/10.1093/bioinformatics/btu031>
- Joos, B., Casey, T.M., Fitzgerald, T.D., & Buttemer, W.A. (1988). Roles of the tent in behavioural thermoregulation of eastern tent caterpillars. *Ecology*, 69, 2004-2011.
- Kamhi, J.F., Arganda, S., Moreau, C.S., & Traniello, J.F.A. (2017). Origins of aminergic regulation of behaviour in complex insect social systems. *Frontiers in Systems Neuroscience*, 11, 1–9.
- Kanehisa, M., & Goto, S. (2000). KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Research*, 28, 27–30. <https://doi.org/10.1093/nar/28.1.27>
- Kapheim, K.M. (2016). Genomic sources of phenotypic novelty in the evolution of eusociality in insects. *Current Opinions in Insect Science*, 13, 24–32. <https://doi.org/10.1016/j.cois.2015.10.009>
- Kapheim, K.M. (2018). Synthesis of Tinbergen's four questions and the future of sociogenomics. *Behavioural Ecology and Sociobiology*, 73, 28. <https://doi.org/10.1007/s00265-018-2626-z>
- Kappeler, P.M. (2019). A framework for studying social complexity. *Behavioural Ecology and Sociobiology*, 73, 13. <https://doi.org/10.1007/s00265-018-2601-8>
- Kato, T., Sawada, H., Yamamoto, T., Mase, K., & Nakagoshi, M. (2006). Pigment pattern formation in the quail mutant of the silkworm, *Bombyx mori*: parallel increase of pteridine biosynthesis and pigmentation of melanin and

- ommochromes. *Pigment Cell and Melanoma Research*, 19(4), 337–345.
<https://doi.org/10.1111/j.1600-0749.2006.00316.x>
- Kocher, S.D, Mallarino, R., Rubin, B.E.R., Yu, D.W., Hoekstra, H.E., & Pierce, N.E. (2018). The genetic basis of social polymorphism in halictid bees. *Nature Communications*, 9, 4338. <https://doi.org/10.1038/s41467-018-06824-8>
- Kojima, K., Ishikawa, Y., & Takanashi, T. (2012). Pupal vibratory signals of a group living beetle that deter larvae: are they mimics of predator cues? *Communicative Integrative Biology*, 5, 262-264. <https://dx.doi.org/10.4161%2Fcib.19886>
- Korb, J. (2009). Termites: An alternative road to eusociality and the importance of group benefits in social insects. In J. Gadau & J. Fewell (Eds.), *Organization of Insect Societies: From Genome to Sociocomplexity* (pp. 128-147). Harvard University Press, Cambridge, Massachusetts.
- Korf, I. (2004). Gene finding in novel genomes. *BMC Bioinformatics*, 5, 59.
<https://doi.org/10.1186/1471-2105-5-59>
- Koutroumpa, F.A., Monsempes, C., François, M.C., De Cian, A., Royer, C., Concordet, J.P., et al. (2016). Heritable genome editing with CRISPR/Cas9 induces anosmia in a crop pest moth. *Scientific Reports*, 6, 1–9. <https://doi.org/10.1038/srep29620>
- Kucharski, R., & Maleszka, R. (2002). Evaluation of differential gene expression during behavioural development in the honeybee using microarrays and northern blots. *Genome Biology*, 3(2), RESEARCH0007.
<https://doi.org/10.1186/gb-2002-3-2research0007>
- Leonhardt, S.D., Menzel, F., Nehring, V., & Schmitt, T. (2016). Ecology and evolution of communication in social insects. *Cell*, 164(6), 1277–1287.
<https://doi.org/10.1016/j.cell.2016.01.035>
- Li, B., & Dewey, C.N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*, 12, 323.
<https://doi.org/10.1186/1471-2105-12-323>
- Liang, Z.S., Nguyen, T., Mattila, H.R., Rodriguez-Zas, S.L., Seeley, T.D., & Robinson, G.E. (2012). Molecular determinants of scouting behaviour in honey bees. *Science*, 335(6073), 1225–1228. <https://doi.org/10.1126/science.1213962>

- Lind, E.M., Jones, M.T., Long, J.D., & Weiss, M.R. (2001). Ontogenetic changes in leaf shelter construction by larvae of *Epargyreus clarus* (Hesperiidae), the Silver spotted Skipper. *Journal of Lepidopterists' Society*, 54, 77–82.
- Liu, X., Liang, P., Gao, X., & Shi, X. (2006). Induction of the cytochrome P450 activity by plant allelochemicals in the cotton bollworm, *Helicoverpa armigera* (Hübner). *Pesticide Biochemistry and Physiology*, 84, 127–134.
<https://doi.org/10.1016/j.pestbp.2005.06.002>
- Livak, K.J., & Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ CT method. *Methods*, 25(4), 402–408.
<https://doi.org/10.1006/meth.2001.1262>
- Low, C. (2008). Seismic behaviours of a leafminer, *Antispila nysaefoliella* (Lepidoptera: Heliozelidae). *Florida Entomologist*, 91, 604–609.
<https://doi.org/10.1653/0015-4040-91.4.604>
- Ma, Z., Guo, X., Lei, H., Li, T., Hao, S., & Kang, L. (2015). Octopamine and tyramine respectively regulate attractive and repulsive behaviour in locust phase changes. *Scientific Reports*, 5, 8036. <https://doi.org/10.1038/srep08036>
- Mahurin, E.J., & Freeberg, T.M. (2009). Chick-a-dee call variation in Carolina chickadees and recruiting flockmates to food. *Behavioural Ecology*, 20, 111–116. <https://doi.org/10.1093/beheco/arn121>
- Maniatis, T., Fritsch, E.F., & Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Markert, M.J., Zhang, Y., Enuameh, M.S., Reppert, S.M., Wolfe, S.A., & Merlin, C. (2016). Genomic access to Monarch migration using TALEN and CRISPR/Cas9 mediated targeted mutagenesis. *G3 (Bethesda, Md.)*, 6(4), 905–915.
<https://doi.org/10.1534/g3.116.027029>
- Matheson, S.M. (2011). *Vibratory mediated spacing in groups of insect larvae (Drepana arcuata, Lepidoptera; Scolytus multistriatus, Coleoptera)* [Published Master's thesis, Carleton University].
https://curve.carleton.ca/system/files/etd/49dcbee3-05d6-4bde-a0d2-82b38c403a36/etd_pdf/450f813e3da0ddcf26dd536f70f703a7/matheson-vibratorymediatedspacingingroupsofinsectlarvae.pdf

- McClellan, Q.C., & Logan, J.A. (1994). Instar determination for the gypsy moth (Lepidoptera: Lymantriidae) based on the frequency distribution of head capsule widths. *Environmental Entomology*, 23, 248–253.
<https://doi.org/10.1093/ee/23.2.248>
- McClure, M., & Despland, E. (2011). Defensive responses by a social caterpillar are tailored to different predators and change with larval instar and group size. *Naturwissenschaften*, 98, 425–434. <https://doi.org/10.1007/s00114-011-0788-x>
- McIver, S., & Beech, M. (1986). Prey finding behaviour and mechanosensilla of larval *Toxorhynchites brevialpilis* Theobald (Diptera: Culicidae). *International Journal of Insect Morphology and Embryology*, 15, 213–225.
[https://doi.org/10.1016/0020-7322\(86\)90059-0](https://doi.org/10.1016/0020-7322(86)90059-0)
- de Mendiburu, F. (2016). Agricolae: statistical procedures for agricultural research. R package version 1.2–4. <https://CRAN.R-project.org/package=agricolae>
- Minet, J., & Scoble, M.J. (1999). The Drepanoid/Geometroid Assemblage. In N.P. Kristensen (Ed.), *Handbook of Zoology: Lepidoptera, Moths and Butterflies. Evolution, Systematics and Biogeography* (Vol.1, pp. 301–305). Berlin: Walter de Gruyter.
- Miura, T. (2004). Proximate mechanisms and evolution of caste polyphenism in social insects: from sociality to genes. *Ecological Research*, 19(2), 141–148.
<https://doi.org/10.1111/j.1440-1703.2003.00618.x>
- Moreira, D., Pereira, A.M., Lopes, A.L., & Coimbra, S. (2020). The best CRISPR/Cas9 versus RNA interference approaches for Arabinogalactan proteins’ study. *Molecular Biology Reports*, 47(3), 2315–2325.
<https://doi.org/10.1007/s11033020-05258-0>
- Mougenot, F., Combe, M., & Jeanson, R. (2012). Ontogenesis and dynamics of aggregation in a solitary spider. *Animal Behaviour*, 84, 391–398.
<https://doi.org/10.1016/j.anbehav.2012.05.008>
- Nelson, C.M., Ihle, K.E., Fondrk, M.K., & Amdam, G.V. (2007). The gene vitellogenin has multiple coordinating effects on social organization. *PLoS Biology*, 5(3), Article e62. <https://doi.org/10.1371/journal.pbio.0050062>

- Ngai, M., & McDowell, M.A. (2017). The search for novel insecticide targets in the post-genomics era, with a specific focus on G-protein coupled receptors. *Memorias Do Instituto Oswaldo Cruz*, 112(1), 1–7.
<https://doi.org/10.1590/007402760160345>
- Novina, C.D., & Sharp, P.A. (2004). The RNAi revolution. *Nature*, 430(6996), 161–164.
<https://doi.org/10.1038/430161a>
- O’Connell, L.A., & Hofmann, H.A. (2011). Genes, hormones, and circuits: An integrative approach to study the evolution of social behaviour. *Frontiers in Neuroendocrinology*, 32(3), 320–335. <https://doi.org/10.1016/j.yfrne.2010.12.004>
- Okamoto, S., Futahashi, R., Kojima, T., Mita, K., & Fujiwara, H. (2008). Catalogue of epidermal genes expressed in the epidermis during larval molt of the silkworm *Bombyx mori*. *BMC Genomics*, 9, 396. <https://doi.org/10.1186/1471-2164-9-396>
- Osborne, K., Robichon, A., Burgess, E., Butland, S., Shaw, R.A., Coulthard, A., et al. (1997). Natural behaviour polymorphism due to a cGMP dependent protein kinase of *Drosophila*. *Science*, 277, 834–836.
<https://doi.org/10.1126/science.277.5327.834>
- Ott, S.R., Verlinden, H., Rogers, S.M., Brighton, C.H., Quah, P.S., Vleugels, R.K., et al. (2012). Critical role for protein kinase A in the acquisition of gregarious behaviour in the desert locust. *Proceedings of National Academy of Science USA*, 109, E381–E387. <https://dx.doi.org/10.1073%2Fpnas.1114990109>
- Packard, A.S. (1890). The life-history of *Drepana arcuata*, with remarks on certain structural features of the larvae and on the supposed dimorphism of *Drepana arcuata* and *Dryopteris rosea*. *Proceedings of Boston Society of Natural History*, 24, 482–493.
- Pearce, S.L., Clarke, D.F., East, P.D., Elfekih, S., Gordon, K.H.J., Jermin, L.S., et al. (2017). Genomic innovations, transcriptional plasticity and gene loss underlying the evolution and divergence of two highly polyphagous and invasive *Helicoverpa* pest species. *BMC Biology*, 15, 63.
<https://doi.org/10.1186/s12915017-0402-6>

- Pescador-Rubio, A., Stanford-Camargo, S.G., Páez-Gerardo, L.E., Ramírez-Reyes, A.J., Ibarra-Jiménez, R.A., & Fitzgerald, T.D. (2011). Trail marking by larvae of the silverspot butterfly *Dione juno huascuma*. *Journal of Insect Science*, 2, 55.
<https://doi.org/10.1673/031.011.5501>
- Peterson, P., Sansko, T.J., Bezuidenhout, C.C., & van den Berg, J. (2019). Transcriptome and differentially expressed genes of *Busseola fusca* (Lepidoptera: Noctuidae) larvae challenged with Cry1Ab toxin. *Gene*, 710, 387–398.
<https://doi.org/10.1016/j.gene.2019.05.048>
- Prokopy, R.J., & Roitberg, B.D. (2001). Joining and avoidance Behaviour in nonsocial insects. *Annual Reviews of Entomology*, 46, 631–635.
<https://doi.org/10.1146/annurev.ento.46.1.631>
- R Core Team. (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
- R Core Team. (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
- Rana, S., Rajurkar, A.B., Kumar, K.K., & Mohankumar, S. (2020). Comparative analysis of chitin synthaseA dsRNA mediated RNA interference for management of crop pests of different families of Lepidoptera. *Frontiers in Plant Science*, 11, 1–12. <https://doi.org/10.3389/fpls.2020.00427>
- Ratchford, S.G., & Eggleston, D.B. (1998). Size and scale-dependent chemical attraction contribute to an ontogenetic shift in sociality. *Animal Behaviour*, 56, 1027–1034.
<https://doi.org/10.1006/anbe.1998.0869>
- Reader, T., & Hochuli, D.F. (2003). Understanding gregariousness in a larval lepidopteran: the roles of host plant, predation, and microclimate. *Ecological Entomology*, 28, 729–737. <https://doi.org/10.1111/j.1365-2311.2003.00560.x>
- Reaume, C.J., & Sokolowski, M.B. (2011). Conservation of gene function in behaviour. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 366(1574), 2100–2110. <https://doi.org/10.1098/rstb.2011.0028>
- Rehan, S.M., Richards, M.H., Adams, M., & Schwarz, M.P. (2014). The costs and benefits of sociality in a facultatively social bee. *Animal Behaviour*, 97, 77–85.
<https://doi.org/10.1016/j.anbehav.2014.08.021>

- Rehan, S.M., & Toth A.L. (2015). Climbing the social ladder: the molecular evolution of sociality. *Trends in Ecology and Evolution*, 30 (7), 426–433.
<https://doi.org/10.1016/j.tree.2015.05.004>
- Rittschof, C.C., & Robinson, G.E. (2014). Genomics: moving behavioural ecology beyond the phenotypic gambit. *Animal Behaviour*, 92, 263–270.
<https://dx.doi.org/10.1016%2Fj.anbehav.2014.02.028>
- Robinson, G.E. (1992). Regulation of division of labor in insect societies. *Annual Review of Entomology*, 37, 637–665.
<https://doi.org/10.1146/annurev.en.37.010192.003225>
- Robinson, G.E., Grozinger, C.M., & Whitfield, C.W. (2005). Sociogenomics: social life in molecular terms. *Nature Reviews Genetics*, 6(4), 257–270.
<https://doi.org/10.1038/nrg1575>
- Robinson, M.D., McCarthy, D.J., & Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, 26(1), 139–40. <https://doi.org/10.1093/bioinformatics/btp616>
- Robinson, G.E., & Vargo, E.L. (1997). Juvenile hormone in adult eusocial Hymenoptera: gonadotropin and behavioural pacemaker. *Archives of Insect Biochemistry & Physiology*, 35, 559–583.
[https://doi.org/10.1002/\(sici\)1520-6327\(1997\)35:4%3C559::aid-arch13%3E3.0.co;2-9](https://doi.org/10.1002/(sici)1520-6327(1997)35:4%3C559::aid-arch13%3E3.0.co;2-9)
- Roe, A.D., Weller, S.J., Baixeras, J., Brown, J., Cummings, M.P., Davis, D.R., et al. (2010). Evolutionary framework for Lepidoptera model systems. In M.R. Goldsmith, & F. Marec (Eds.), *Molecular Biology and Genetics of the Lepidoptera* (pp. 1–24). Boca Raton: CRC Press.
- Roeder, T. (1999). Octopamine in invertebrates. *Progress in Neurobiology*, 59, 533–561.
[https://doi.org/10.1016/s0301-0082\(99\)00016-7](https://doi.org/10.1016/s0301-0082(99)00016-7)
- Roeder, T. (2005). Tyramine and octopamine: ruling behaviour and metabolism. *Annual Review of Entomology*, 50, 447–477.
<https://doi.org/10.1146/annurev.ento.50.071803.130404>
- Rose, A.H., & Lindquist, O.H. (1997). Insects of eastern hardwood trees. Canadian Forestry Service, Ottawa, Forestry Technical Report 29

- Rosi-Denadai, C. (2018). *How do caterpillars detect vibration? Proleg sensory hairs as vibration receptors in Drepana arcuata (Drepanidae) and Trichoplusia ni (Noctuidae)*. Dissertation, Carleton University. [Published Master's thesis, Carleton University].
<https://curve.carleton.ca/8de0920f-b83f-40d8-aa81-e8b2f44e9cda>
- Rubenstein, D.R., & Hofmann, H.A. (2015). Proximate pathways underlying social behaviour. *Current Opinion in Behavioural Sciences*, 6, 154–159.
<https://doi.org/10.1016/j.cobeha.2015.11.007>
- Ruf, C., & Fiedler, K. (2002). Tent-based thermoregulation in social caterpillars of *Eriogaster lanestris* (Lep., Lasiocampidae): behavioural mechanisms and physical features of the tent. *Journal of Thermal Biology*, 27, 493–501.
- Scoble, M.J. (1992). *The Lepidoptera: Form, Function and Diversity*. The Natural History Museum & Oxford University Press, London.
- Scott, J.L. (2012). *The evolutionary origins of vibratory signals in Drepanidae caterpillars: A comparative study on morphology, phylogenetics and behaviour*. [Published Doctoral Dissertation, Carleton University].
https://curve.carleton.ca/system/files/etd/6efb394c-4510-4087-bc895c398d818b28/etd_pdf/8d582718c64fcf526d43394864b5b5fe/scotttheevolutionaryoriginsofvibratorysignalsin.pdf
- Scott, J.L., Kawahara, A.Y., Skevington, J.H., Yen, S.H., Sami, A., Smith, M.L., et al. (2010a). The evolutionary origins of ritualized acoustic signals in caterpillars. *Nature Communications*, 1, 1–9. <https://doi.org/10.1038/ncomms1002>
- Scott, J.L., Matheson, S.M., & Yack, J.E. (2010b). Variation on a theme: vibrational signalling in caterpillars of the rose hook-tip moth, *Oreta rosea*. *Journal of Insect Science*, 10, 15. <https://doi.org/10.1673/031.010.5401>
- Scott, J.L., & Yack, J.E. (2012). Vibratory territorial signals in caterpillars of the poplar lutestring, *Tethea or* (Lepidoptera: Drepanidae). *European Journal of Entomology*, 109, 411–417.
- Schulz, D.J., Barron, A.B., & Robinson, G.E. (2002). A role for octopamine in honey bee division of labor. *Brain, Behaviour and Evolution*, 60(6), 350–359.
<https://doi.org/10.1159/000067788>

- Seeley, T.D. (1995). *The Wisdom of the Hive*. Harvard University Press, Cambridge
- Seymour, R.S. (1974). Convective and evaporative cooling in sawfly larvae. *Journal of Insect Physiology*, 20, 2447–2457.
- Shell, W.A., & Rehan, S.M. (2017). Behavioural and genetic mechanisms of social evolution: insights from incipiently and facultatively social bees. *Apidologie*, 49, 13–30. <https://doi.org/10.1007/s13592-017-0527-1>
- Shell, W.A., & Rehan, S.M. (2019). Social modularity: conserved genes and regulatory elements underlie caste-antecedent behavioural states in an incipiently social bee. *Proceedings of the Royal Society B: Biological Sciences*, 286(1916), 20191815. <https://doi.org/10.1098/rspb.2019.1815>
- Sillén-Tullberg, B. (1988). Evolution of gregariousness in aposematic butterfly larvae – a phylogenetic analysis. *Evolution*, 42, 293–305.
- Simão, F.A., Waterhouse, R.M., Ioannidis, P., Kriventseva, E.V., & Zdobnov, E.M. (2015). BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics (Oxford, England)*, 31(19), 3210–3212. <https://doi.org/10.1093/bioinformatics/btv351>
- Siomi, H., & Siomi, M.C. (2009a). RISC hitches onto endosome trafficking. *Nature Cell Biology*, 11(9), 1049–1051. <https://doi.org/10.1038/ncb0909-1049>
- Siomi, H., & Siomi, M.C. (2009b). On the road to reading the RNA-interference code. *Nature*, 457(7228), 396–404. <https://doi.org/10.1038/nature07754>
- Slocombe, K.E., Kaller, T., Turman, L., Townsend, S.W., Papworth, S., Squibbs, P., et al. (2010). Production of food-associated calls in wild chimpanzees is dependent on the composition of the audience. *Behavioural Ecology and Sociobiology*, 64, 1959–1966. <https://doi.org/10.1007/s00265-010-1006-0>
- Spindler, S.R., Mote, P.L., Li, R., Dhahbi, J.M., Yamakawa, A., Flegel, J.M., et al. (2013). β 1-Adrenergic receptor blockade extends the life span of *Drosophila* and long-lived mice. *Age*, 35(6), 2099–2109. <https://doi.org/10.1007/s11357-0129498-3>
- Stamp, N.E. (1980). Egg deposition patterns in butterflies: Why do some species cluster their eggs rather than deposit them singly? *The American Naturalist*, 115, 367–380.

- Stehr, F.W. (1987). Order Lepidoptera. In F. W. Stehr (Ed.), *Immature Insects* (Vol. 1, pp. 288–596). Kendall/Hunt, Dubuque.
- Stern, M. (1999). Octopamine in the locust brain: Cellular distribution and functional significance in an arousal mechanism. *Microscopy Research and Technique*, 45(3), 135–141.
[https://doi.org/10.1002/\(sici\)1097-0029\(19990501\)45:3%3C135::aid-jemt1%3E3.0.co;2-g](https://doi.org/10.1002/(sici)1097-0029(19990501)45:3%3C135::aid-jemt1%3E3.0.co;2-g)
- Surlykke, A., Yack, J.E., Spence, A.J., & Hasenfuss, I. (2003). Hearing in hooktip moths (Drepanidae, Lepidoptera). *Journal of Experimental Biology*, 206, 2653–2663. <https://doi.org/10.1242/jeb.00469>
- Suzuki, T.N. (2012). Long-distance calling by the willow tit, *Poecile montanus*, facilitates formation of mixed-species foraging flocks. *Ethology*, 118, 10–16.
<https://doi.org/10.1111/j.1439-0310.2011.01982.x>
- Szipl, G., Boeckle, M., Wascher, C.A.F., & Spreafico, M. (2015). With whom to dine? Ravens' responses to food-associated calls depend on individual characteristics of the caller. *Animal Behaviour*, 99, 33–42.
<https://doi.org/10.1016/j.anbehav.2014.10.015>
- Tarailo-Graovac, M., & Chen, N. (2009). Using RepeatMasker to identify repetitive elements in genomic sequences. *Current Protocols in Bioinformatics*, 25, 4.10.1–4.10.14.
<https://doi.org/10.1002/0471250953.bi0410s25>
- Tatusov, R.L., Galperin, M.Y., Natale, D.A., & Koonin, E.V. (2000). The COG database: a tool for genome-scale analysis of protein functions and evolution. *Nucleic acids research*, 28(1), 33–36.
<https://doi.org/10.1093/nar/28.1.33>
- Tautz, J., Roces, F., & Hölldobler, B. (1995). Use of a sound-based vibratome by leaf cutting ants. *Science*, 267, 84–87.
- Terenius, O., Papanicolaou, A., Garbutt, J.S., Eleftherianos, I., Huvenne, H., Kanginakudru, et al. (2011). RNA interference in Lepidoptera: an overview of successful and unsuccessful studies and implications for experimental design.

- Journal of Insect Physiology*, 57(2), 231–245.
<https://doi.org/10.1016/j.jinsphys.2010.11.006>
- Thrasher, A., Musgrave, Z., Kachmarck, B., Thain, D., & Emrich, S. (2014). Scaling up genome annotation using MAKER and work queue. *International Journal of Bioinformatics Research and Applications*, 10(4-5), 447–460.
<https://doi.org/10.1504/IJBRA.2014.062994>
- Tinbergen, N. (1963). On aims and methods in ethology. *Zeitschrift für Tierpsychologie*, 20, 410–433.
- Towns, J., Cockerill, T., Dahan, M., Foster, I., Gaither, K., Grimshaw, A., et al. (2014). XSEDE: Accelerating Scientific Discovery. *Computing in Science & Engineering*, 1(5), 62–74.
- Travassos, M.A., & Pierce, N.E. (2000). Acoustics, context and function of vibrational signalling in a lycaenid butterfly-ant mutualism. *Animal Behaviour*, 60, 13–26.
<https://doi.org/10.1006/anbe.1999.1364>
- Triant, D.A., Cinel, S.D., & Kawahara, A.Y. (2018). Lepidoptera genomes: current knowledge, gaps and future directions. *Current Opinions in Insect Science*, 25, 99–105. <https://doi.org/10.1016/j.cois.2017.12.004>
- Tribble, W., Olivós-Cisneros, L., McKenzie, S.K., Saragosti, J., Chang, N.C., Matthews, B.J., et al. (2017). orco mutagenesis causes loss of antennal lobe glomeruli and impaired social behaviour in ants. *Cell*, 170, 727–735.e10.
<https://doi.org/10.1016/j.cell.2017.07.001>
- Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., & Leunissen, J.A. (2007). Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research*, 35, W71–W74. <https://doi.org/10.1093/nar/gkm306>
- Velásquez, Y., & Viloria, A. (2010). Instar determination of the neotropical beetle *Oxelytrum discicollae* (Coleoptera: Silphidae). *Journal of Medical Entomology*, 47, 723–726.
- Verlinden, H., Vleugels, R., Marchal, E., Badisco, L., Tobback, J., Pflüger, H. J., et al. (2010). The cloning, phylogenetic relationship and distribution pattern of two new putative GPCR-type octopamine receptors in the desert locust (*Schistocerca*

- gregaria*). *Journal of Insect Physiology*, 56(8), 868–875.
<https://doi.org/10.1016/j.jinsphys.2010.03.003>
- Virant-Doberlet, M., & Čokl, A. (2004). Vibrational communication in insects. *Neotropical Entomology*, 33(2), 121–134.
<https://doi.org/10.1590/S1519566X2004000200001>
- Virant-Doberlet, M., Kuhelj, A., Polajnar, J., & Šturm, R. (2019). Predator-prey interactions and eavesdropping in vibrational communication networks. *Frontiers in Ecology and Evolution*, 7, 203. <https://doi.org/10.3389/fevo.2019.00203>
- Vulinec, K. (1990). Collective security: Aggregation by insects as a defense. In D. L. Evans & J. O. Schmidt (Eds.), *Insect Defenses: Adaptive Mechanisms and Strategies of Prey and Predators* (251–288). State University of New York Press, Albany, New York.
- Wada-Katsumata, A., Yamaoka, R., & Aonuma, H. (2011). Social interactions influence dopamine and octopamine homeostasis in the brain of the ant *Formica japonica*. *Journal of Experimental Biology*, 214(10), 1707–1713.
<https://doi.org/10.1242/jeb.051565>
- Wallbank, R.W., Baxter, S.W., Pardo-Diaz, C., Hanly, J.J., Martin, S.H., Mallet, J., et al. (2016). Evolutionary novelty in a butterfly wing pattern through enhancer shuffling. *PLoS Biology*, 14(1), e1002353.
<https://doi.org/10.1371/journal.pbio.1002353>
- Wang, M., Zhang, S., Shi, Y., Yang, Y., & Wu, Y. (2020). Global gene expression changes induced by knockout of a protease gene cluster in *Helicoverpa armigera* with CRISPR/Cas9. *Journal of Insect Physiology*, 122, 104023.
<https://doi.org/10.1016/j.jinsphys.2020.104023>
- Ward, A., & Webster, M. (2016). *Sociality: The behaviour of Group-Living Animals*. Springer. <https://doi.org/10.1007/978-3-319-28585-6>
- Warrant, E.J., Kelber A., & Kristensen N.P. (2003). Eyes and vision. In N. P. Kristensen (Ed.), *Handbook of Zoology Lepidoptera, Moths and Butterflies: Morphology, Physiology and Development* (Part 36, Vol. 2, pp. 325–359). Berlin/New York: Walter de Gruyter.

- Weed, A.S. (2010). Benefits of larval group feeding by *Chrysolina aurichalcea asclepiadis* on *Vincetoxicum*: improved host location or feeding facilitation? *Entomologia Experimentalis et Applicata*, 137(3), 220-228
<https://doi.org/10.1111/j.1570-7458.2010.01057.x>
- Weiss, M.R. (2003). Good housekeeping: why do shelter-dwelling caterpillars fling their frass?. *Ecology Letters*, 6(4), 361-370.
<https://doi.org/10.1046/j.1461-0248.2003.00442.x>
- Wirth, R., Herz, H., Ryel, R.J., Beyschlag, W., Hölldobler, B. & Molnar, Z.D. (2003). Herbivory of leaf-cutting ants: a case study on *Atta colombica* in the tropical rainforest of Panama. *Ecological Studies*, 164, 1–230.
- Whitfield, C.W., Cziko, A.M., & Robinson, G.E. (2003). Gene expression profiles in the brain predict behaviour in individual honey bees. *Science*, 302(5643), 296-299. <https://doi.org/10.1126/science.1086807>
- Wilson, E.O. (1971). *The Insect Societies*. Cambridge, ME: Belknap Press of Harvard University Press.
- Wilson, E.O. (1985). The sociogenesis of insect colonies. *Science*, 228(4707), 1489-1495. <https://doi.org/10.1126/science.228.4707.1489>
- Wu, Q., Wen, T., Lee, G., Park, J.H., Cai, H.N., & Shen, P. (2003). Developmental control of foraging and social behaviour by the *Drosophila* neuropeptide Y-like system. *Neuron*, 39(1), 147-161. [https://doi.org/10.1016/s0896-6273\(03\)00396-9](https://doi.org/10.1016/s0896-6273(03)00396-9)
- Xu, J., Wang, X.F., Chen, P., Liu, F.T., Zheng, S.C., Ye, H., et al. (2016). RNA interference in moths: mechanisms, applications, and progress. *Genes*, 7(10), 88
<https://doi.org/10.3390/genes7100088>
- Xu, L., Li, L., Yang, P., & Ma, Z. (2017). Calmodulin as a downstream gene of octopamine-OAR $\alpha 1$ signalling mediates olfactory attraction in gregarious locusts. *Insect Molecular Biology*, 26(1), 1-12. <https://doi.org/10.1111/imb.12266>
- Yack, J.E. (2016). Vibrational signalling. In G.S. Pollack, A.C. Mason, R.R. Fay, & A.N. Popper (Eds.), *Springer Handbook of Auditory Research: Insect Hearing* (pp. 99-123). New York, NY: Springer.

- Yack, J.E., Gill, S., Drummond-Main, C., & Sherratt, T.N. (2014). Residency duration and shelter quality influence vibratory signalling displays in a territorial caterpillar. *Ethology*, 120(4), 354-364. <https://doi.org/10.1111/eth.12210>
- Yack, J.E., Smith, M.L., & Weatherhead, P.J. (2001). Caterpillar talk: acoustically mediated territoriality in larval Lepidoptera. *Proceedings of the National Academy of Sciences*, 98(20), 11371-11375. <https://doi.org/10.1073/pnas.191378898>
- Yack, J.E., & Yadav, C. (2021). Vibratory sensing and communication in caterpillars. In P. Hill (Ed.), *Biotremology: Physiology, Ecology, and Evolution* (In Press).
- Yadav, C., Guedes, R.N.C., Matheson, S.M., Timbers, T.A., & Yack, J.E. (2017). Invitation by vibration: recruitment to feeding shelters in social caterpillars. *Behavioural Ecology and Sociobiology*, 71(3), 51. <https://doi.org/10.1007/s00265-017-2280-x>
- Yadav, C., Smith, M.L., & Yack, J.E. (2020). Transcriptome analysis of a social caterpillar, *Drepana arcuata*: De novo assembly, functional annotation and developmental analysis. *PLoS ONE*, 15(6), Article e0234903. <https://doi.org/10.1371/journal.pone.0234903>
- Yadav, C., & Yack, J.E. (2018). Immature stages of the masked birch caterpillar, *Drepana arcuata* (Lepidoptera: Drepanidae) with comments on feeding and shelter building. *Journal of Insect Science*, 18(1), 18. <https://doi.org/10.1093/jisesa/iey006>
- Yan, H., Opachaloemphan, C., Mancini, G., Yang, H., Gallitto, M., Mlejnek, J., et al. (2017). An engineered orco mutation produces aberrant social behaviour and defective neural development in ants. *Cell*, 170(4), 736-747. <https://doi.org/10.1016/j.cell.2017.06.051>
- Ye, Z.F., Liu, X.L., Han, Q., Liao, H., Dong, X.T., Zhu, G.H., et al. (2017). Functional characterization of PBP1 gene in *Helicoverpa armigera* (Lepidoptera: Noctuidae) by using the CRISPR/Cas9 system. *Scientific Reports*, 7(1), 8470 8470. <https://doi.org/10.1038/s41598-017-08769-2>

- Zalucki, M.P., Clarke, A.R., & Malcolm, S.B. (2002). Ecology and behaviour of first instar larval Lepidoptera. *Annual Review of Entomology*, 47(1), 361-393.
<https://doi.org/10.1146/annurev.ento.47.091201.145220>
- Zayed, A., & Kent, C. (2015). *Genomics, Physiology and Behaviour of Social Insects* (1st ed). Cambridge, England: Academic Press.
- Zeng, R. S., Wen, Z., Niu, G., Schuler, M.A., & Berenbaum, M.R. (2007). Allelochemical induction of cytochrome P450 monooxygenases and amelioration of xenobiotic toxicity in *Helicoverpa zea*. *Journal of Chemical Ecology*, 33(3), 449. <https://doi.org/10.1007/s10886-006-9238-1>
- Zhang, L., & Reed, R.D. (2017). A Practical Guide to CRISPR/Cas9 Genome Editing in Lepidoptera. In T. Sekimura, & H. F. Nijhout (Eds.), *Diversity and Evolution of Butterfly Wing Patterns: An Integrative Approach* (pp. 155–172). Singapore: Springer. https://doi.org/10.1007/978-981-10-4956-9_8
- Zhang, S.V., Zhuo, L., & Hahn, M.W. (2016). AGOUTI: improving genome assembly and annotation using transcriptome data. *GigaScience*, 5(1), s13742-016.
<https://doi.org/10.1186/s13742-016-0136-3>
- Zimin, A.V., Marçais, G., Puiu, D., Roberts, M., Salzberg, S.L., & Yorke, J.A. (2013). The MaSuRCA genome assembler. *Bioinformatics*, 29(21), 2669-2677.
<https://doi.org/10.1093/bioinformatics/btt476>